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Title: Cannabis sativa and Humulus lupulus essential oils as novel control tools against the invasive mosquito Aedes albopictus and fresh water snail Physella acuta

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Abstract: Over the past several decades, there has been a resurgence of interest in industrial hemp (Cannabis sativa L., Cannabaceae) cultivation. Besides fibre, seeds and oil, hemp contains high quantity of essential oil (EO). Hop (Humulus lupulus L., Cannabaceae) is a high-climbing, perennial vine, largely utilized in the brewing industry to add flavour and bitterness to beer. While it is known that hop also contains α- and ß-acids, and terpenes that have been found to be toxic, anti-feedant, and repellent for insects and mites, little is known about the bioactivity against problematic species of the hemp essential oil. In this study, the chemical composition of the EOs from C. sativa and H. lupulus was evaluated by GC-MS, and their acute toxicity was assessed against, the Asian tiger mosquito Aedes albopictus (Skuse) (Diptera Culicidae) and, the freshwater bladder snail Physella acuta (Draparnaud) (Mollusca Physidae), two problematic invasive species. Furthermore, we evaluated the toxicity of both EOs against a non-target insect, the mayfly Cloeon dipterum L. (Ephemeroptera Baetidae). Both EOs were toxic against the three tested species. The most effective EO was the C. sativa, able to kill 100% of P. acuta snails starting from 100 µL L-1. C. sativa LC50 were 301.560, 282.174 and, 35.370 µL L-1, while H. lupulus LC50 were 330.855, 219.787 and, 118.653 µL L-1 against A. albopictus, C. dipterum and P. acuta, respectively. Relative median potency analysis showed that the C. sativa EO was more toxic than H. lupulus against A. albopictus and P. acuta, while H. lupulus was more toxic than C. sativa EO against C. dipterum. The most susceptible species to the two EOs was P. acuta, while A. albopictus resulted the least susceptible one.

Graphical Abstract

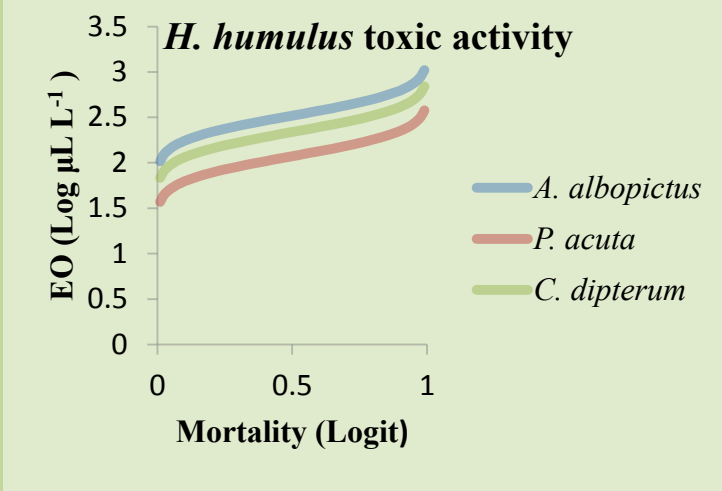
Cannabis sativa

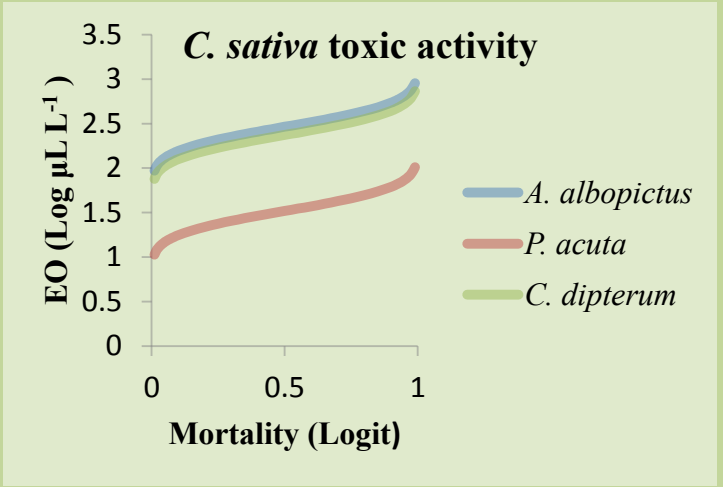
Humulus lupulus

Aedes albopictus

Physella acuta

Cloeon dipterum





HIGHLIGHTS

- Hemp and hop EOs are mosquitocidal and molluscicidal
- Hemp essential oil was very effective in killing the invasive snail *Physella acuta*
- The non-target mayfly, was less affected than the target *P. acuta* by hemp EO
- EOs based pesticides could be a new industrial use of hemp and hop

Cannabis sativa and *Humulus lupulus* essential oils as novel control tools against the invasive mosquito *Aedes albopictus* and fresh water snail *Physella acuta*

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ABSTRACT

Over the past several decades, there has been a resurgence of interest in industrial hemp (*Cannabis sativa* L., Cannabaceae) cultivation. Besides fibre, seeds and oil, hemp contains high quantity of essential oil (EO). Hop (*Humulus lupulus* L., Cannabaceae) is a high-climbing, perennial vine, largely utilized in the brewing industry to add flavour and bitterness to beer. While it is known that hop also contains α- and ß-acids, and terpenes that have been found to be toxic, anti-feedant, and repellent for insects and mites, little is known about the bioactivity against problematic species of the hemp ~~essential oil~~EO. In this study, the chemical composition of the EOs from *C. sativa* and *H. lupulus* was evaluated by GC-MS, and their acute toxicity was assessed against, the Asian tiger mosquito *Aedes albopictus* (Skuse) (Diptera Culicidae) and, the freshwater bladder snail *Physella acuta* (Draparnaud) (Mollusca Physidae), two problematic invasive species. Furthermore, we evaluated the toxicity of both EOs against a non-target insect, the mayfly *Cloeon dipterum* L. (Ephemeroptera Baetidae). Both EOs were toxic against the three tested species. The most effective EO was the *C. sativa*, able to kill 100% of *P. acuta* snails starting from 100 µL L^{-1}. *C. sativa* LC_{50} were 301.560, 282.174 and, 35.370 µL L^{-1}, while *H. lupulus* LC_{50} were 330.855, 219.787 and, 118.653 µL L^{-1} against *A. albopictus*, *C. dipterum* and *P. acuta*, respectively. Relative median potency analysis showed that the *C. sativa* EO was more toxic than *H. lupulus* against *A. albopictus* and *P. acuta*, while *H. lupulus* was more toxic than *C. sativa* EO against *C. dipterum*. The most susceptible species to the two EOs was *P. acuta*, while *A. albopictus* resulted the least susceptible one.

Keywords: ~~Arbovirus; Filariasis~~Essential oils; GC-MS, Mosquitoes, Invasive ~~species~~snails, Botanical p~~P~~esticides; Non-target aquatic organisms.

1. Introduction

Hemp (*Cannabis sativa* L.) is not only one of the oldest known medicinal plants, but it has been also largely cultivated for centuries for fibre and seeds. In many countries, hemp cultivation has been prohibited because of its content of psychotropic chemical components (tetrahydrocannabinol, also known as THC). However, it has been pointed out that hemp medicinal properties could be useful to treat numerous diseases and for pain relief and that the beneficial health effects outweigh the psychotropic properties of *Cannabis*. This has recently led some countries (i.e. Holland, Germany, Romania, Slovenia, Israel, USA and Italy) to legalise this plant or its derivatives for medicinal purpose. Besides, from 2001, the European Commission allowed the hemp cultivation with less than 0.2 % THC. All these facts determined an increasing interest in hemp cultivation and in the use of the plant-derived raw materials such as the hemp fibre, that can be used in the production of specialty papers, and the hemp seeds that can be used as a food and feed and contain an oil useful for manufacturing printer ink, for wood preservation, and production of soaps and detergents (Callaway, 2004~~; Ranalli and Venturi, 2004~~). Moreover, hemp flowers and upper leaves also contain an essential oil (EO) used as a scent in perfumes cosmetics, soaps, candles and as flavouring in foods. Interestingly, hemp EO has also been shown to be toxic to mosquitoes larvae (Thomas et al., 2000) and recently, to have antimicrobial (Verma et al., 2014) and nematicidal (Mukhtar et al., 2013) properties.

Hop (*Humulus lupulus* L.), another member of the small Cannabaceae family, is a natural component of riverside wetland forests of the temperate northern hemisphere~~ (Prieditis, 1997)~~. Hop, ~~has been~~ cultivated since ancient times ~~and~~ is mainly used as a bittering agent in the beer brewing process~~ (Chadwick et al., 2006)~~. H~~In the h~~op female strobilus inflorescences (hops or cones) content more than 1000 chemical compounds ~~have been identified~~ and the hop extracts have shown a strong bioactivity as antimicrobial, estrogenic and, anticancerogenic (Farago et al., 2009~~; Wang et al., 2008~~).

In particular, hop α- and ß-acids, and terpenes have been found to be toxic, anti-feeding, and repellent for ~~several~~ insects and mites of economic importance (Bedini et al., 2015; DeGrandi-Hoffman et al., 2012~~; Gökçe et al., 2009; Powell et al., 1997~~).

The Asian tiger mosquito *Aedes albopictus* (Skuse) (Diptera Culicidae), due to its ecological and physiological plasticity ~~(Yamany et al., 2012)~~, is acknowledged as the most invasive mosquito species worldwide ~~(Benedict et al., 2007; Caminade et al., 2012)~~. Moreover, because of its aggressive daytime human-biting behaviour and its ability to transmit many pathogens and parasites, including dengue, West Nile, Japanese encephalitis, yellow fever and, chikungunya ~~(Mehlhorn, 2011; Benelli, 2015a)~~, it represents a key threat for millions of people worldwide (Paupy et al., 2009).

The freshwater pan-pulmonate snail *Physella acuta* (Draparnaud) (Mollusca Physidae) is another problematic invasive species that shares the same habitats of the *A. albopictus* larvae and it is considered a plague in rice fields (Banha et al., 2014). Like the tiger mosquito, also *P. acuta* is a species of medical importance, mainly due to the fact that it is an intermediate host for trematode and nematode human parasites (~~Faltýnková, 2005;~~ Faltýnková and Haas, 2006~~; Hai et al., 2009; Toledo et al., 1999~~).

Nowadays, pests are largely controlled by synthetic pesticides. However, the continuous use of organophosphates and insect growth regulators has caused the rising of resistant mosquito strains ~~(Benelli 2015b)~~. Besides, currently employed molluscicides are limited in number, expensive and also have negative effects on human health and the environment (~~Hemingway and Ranson, 2000; Lees et al., 2014; Madsen, 1990;~~ Severini et al., 1993~~; Sun et al., 2011~~).

In this scenario, there is a growing interest for alternative eco-friendly control tools for pest management ~~(Duke et al., 2010)~~. Natural products often fill these needs. In particular, in recent years, ~~essential oils~~EOs of aromatic plants received a great attention for pest control purposes ~~(Benelli 2015b, 2015c; Benelli et al., 2013, Conti et al., 2010; 2012a; 2012b)~~, since they are often characterized by low

toxicity towards mammalians (Regnault-Roger et al., 2012). To be acceptable, however, natural pesticides must be not only highly toxic against the targeted pests but they also should not have strong toxicity against non-target organisms.

In the present work, hemp and hops ~~essential oils~~EOs were chemically analysed and their acute toxicities evaluated against larvae of *A. albopictus* and against adults of *P. acuta*. The toxicity of *C. sativa* and *H. lupulus* ~~essential oils~~EOs was also assessed against the mayfly *Cloeon dipterum* L. (Ephemeroptera Baetidae) a non-target aquatic organism sharing the same habitat of mosquito larvae and *Physella* snails.

2. Materials and methods

2.1 EOs~~sential oil~~ extraction and GC-MS analyses

C. sativa EO was purchased from Assocanapa srl (Torino, Italy), while~~.~~ *H. lupulus* cv Cascade cones were obtained from Mr. Malt srl (Udine, Italy). The plant material (3x50 g) ~~were~~ was hydro-distilled in a Clevenger-type apparatus for 2 h. Gas chromatography-electron impact mass spectroscopy (GC-EIMS) analyses were performed with a Varian CP-3800 gas chromatograph, equipped with a HP-5 capillary column (30 m×0.25 mm; coating thickness 0.25 µm) and a Varian Saturn 2000 ion trap mass detector. Analytical conditions were injector and transfer line temperatures at 220 and 240 °C, respectively, oven temperature programmed from 60 to 240 °C at 3 °C/min, carrier gas helium at 1 mL/min, injection of 0.2 µL (10 % hexane solution), and split ratio 1:30. All the injections were performed in triplicate. For *Cannabis oil* three independent samples were prepared from the same ~~essential oil~~EO, while for hop the analyses were performed on each hydrodistilled sample. Constituents identification was based on comparison of retention times with those of authentic samples, comparing

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their LRIs with the series of *n*-hydrocarbons and using computer matching against commercial (NIST 98 and ADAMS) and home-made library mass spectra (built up from pure substances and components of known oils and mass spectra literature data) (Adams, 1995~~; Davies, 1990; Massada, 1976; Jennings and Shibamoto, 1980; Swigar and Silverstein, 1981~~).

2.2 Insect cultures and rearing conditions

2.2.1 Aedes albopictus

Larvae of *A. albopictus* originated from field-collected eggs, deposited by wild females on bars of masonite placed outdoors in dark vases containing tap water. Egg batches were collected daily and kept moist for 24 h. Then, they were placed in laboratory conditions (25 ± 1 °C, 45 ± 5 % relative humidity, natural summer photoperiod) in 250 cc beakers and submerged in tap water for hatching. Newly emerged larvae were single reared in 50 cc vials, with tap water and a small amount of cat food until they reached the fourth instar stage, when they were used for the bioassay. ~~(Conti et al., 2012a; 2012b).~~

2.2.2 Physella acuta

Adult snails of *P. acuta* (length 6.1 mm ± 0.2 m) were collected from field water tanks at the Department of Agriculture, Food and Environment in July 2014, then transferred to laboratory conditions (24 ± 1 °C; 50 ± 5 % RH, natural photoperiod) and identified to specific level through molecular characterization (Benelli et al., 2015b). *P. acuta* snails were maintained in polyethylene aquaria (40, 30, 30 cm) containing about 10 L of tap water (21 ± 1 °C, pH 7.3-7.5). Three times per week, the aquaria were cleaned, removing excrements and dead snails. Lettuce leaves were used as food. Only adult snails were used for bioassays.

2.2.3 Cloeon dipterum

Cloeon dipterum nymphs were collected from field water tanks at the Department of Agriculture, Food and Environment, identified at specific level following the keys reported in Grandi (1960), then reared in laboratory conditions (24 ± 1 °C; 50 ± 5 % R.H.; natural photoperiod) in polyethylene aquaria (40, 30, 30 cm) containing about 10 L of tap water and fed with leaf litter. Late instars nymphs (length 3.9 mm ± 0.2 m) were used for bioassays.

2.3 Toxicity bioassays

2.3.1 Toxicity of ~~essential oils~~EOs against Aedes albopictus

Three groups of 20 larvae (fourth instar) were isolated in 250-mL beakers and exposed for 24 h to dosages ranging from 25 to 500 µL L^{-1} of *C. sativa* and *H. lupulus* ~~essential oils~~EOs. Each tested product was dissolved in tap water containing 0.025 % of Tween 80. Tap water with 0.025 % of Tween 80 was used as control. Mortality was checked after 24 h and reported as an average of three replicates; data were also used to calculate the LC_{50} value (WHO, 1981).

2.3.2. Toxicity of ~~essential oils~~EOs against Physella acuta

Three groups of 20 specimens of *P. acuta* were isolated in 250-mL beakers and exposed for 24 h to dosages ranging from 25 to 500 µL L^{-1} of *C. sativa H. lupulus* ~~essential oils~~EOs in tap water containing 0.025 % of Tween 80. The beakers were covered with a net to prevent snails from falling out. Snails were not fed during this period. At the end of the exposure period, mortality was checked. Control experiments were executed similarly and simultaneously as the treatments. 250 mL beakers with the same number of *P. acuta* individuals (three replicates) and tap water with 0.025 % of Tween 80 were used as control. Both in treatment and control experiments, mortality was confirmed by the absence of

heartbeat and lack of reaction by probing the snails with a needle to elicit typical withdrawal movements (~~Lahlou, 2004;~~ Teixeira et al., 2012). *P. acuta* mortalities were reported as an average of three replicates, data were also used to calculate the LC_{50} value.

2.3.3 Toxicity of ~~essential oils~~EOs against the non-target mayfly Cloeon dipterum

Three groups of ten *C. dipterum* nymphs were isolated in 250-mL beakers and exposed for 24 h to dosages ranging from 50 to 500 µL L^{-1} of *C. sativa* and *H. lupulus* ~~essential oils~~EOs in tap water containing 0.025 % of Tween 80. 250 mL beakers with the same number of *C. dipterum* individuals (three replicates) and tap water with 0.025 % of Tween 80 were used as control. Mortality in treated specimens was recorded after 24 h, at the end of the test, during which no food was given to the specimens. ~~(Benelli et al., 2015b).~~ *C. dipterum* mortalities were reported as an average of three replicates, data were also used to calculate the LC_{50} value.

2.4 Data analysis

Mortality data were transformed into arcsine/proportion values before statistical analysis. Since no mortality was registered in the control treatment, the mortality percentage rates were not corrected. Data were processed by a general linear model (GLM) with three factors, the tested invertebrate species, the EO and the EO dosage. Averages were separated by Tukey's b post hoc test. $P<0.05$ was used for the significance of differences between means.

Median lethal concentration (LC_{50}) was calculated by Log-probit regression. ~~(Finney, 1971).~~ Significant differences between LC_{50} values were determined by estimation of confidence intervals of the relative median potency (rmp). Differences among LC_{50} values were judged to be statistically significant when 1.0 was not found in the 95% confidence interval of relative median potency. All the

analyses were performed by the SPSS 22.0 software (SPSS Inc., Chicago, IL, USA).

3 Results

3.1 ~~Essential oils~~EOs extraction and GC-MS analysis

GC-MS analyses on the ~~essential oils~~EOs obtained from the aerial parts of *C. sativa* and from cones of *H. lupulus* led to the identification of, respectively, 34 and 38 compounds, representing 97.6 and 99.7% of the whole *C. sativa* and *H. lupulus* oils, respectively (Table 1). ~~Essential oil~~EO yield from hop was 0.11 % (w/w). The main chemical class of both ~~essential oils~~EOs components were monoterpene hydrocarbons (57.2 % for *C. sativa* and 70.4 % for *H. lupulus*) (Table 2). Myrcene, *β*-caryophyllene and terpinolene were the most abundant chemical components of *C. sativa* ~~essential oil~~EO (22.9, 18.7 and 12.0 %, respectively) while in *H. lupulus* ~~essential oil~~EO the major constituents were myrcene, α-humulene and β-caryophyllene (68.0, 13.3 and 3.7 %, respectively) (Table 1).

3.2 Toxicity bioassays

Both EOs showed a clear toxic activity against the three species *A. albopictus*, *C. dipterum* and *P. acuta*. *C. sativa* LC_{50} values were 301.560, 282.174 and, 35.370 µL L^{-1} while, *H. lupulus* LC_{50} values were 330.855, 219.787 and, 118.653 µL L^{-1} against *A. albopictus*, *C. dipterum* and *P. acuta*, respectively (Table 3). Univariate GLM test showed no significant differences between ~~essential oils~~EOs toxicity (F=1.310, d.f. = 1; P=0.255), whereas a significant effect of the tested species (F=281.446, d.f. = 2; P<0.0001) and ~~essential oil~~EO dosage (F=266.005, d.f. = 7; P<0.0001) was found. In addition, the interactions of species * oil (F=76.010, d.f. = 2; P<0.0001), oil * dosage

(*F*=15.481, d.f. = 7; *P*<0.0001), species * dosage (*F*=15.657, d.f. = 14; *P*<0.0001) and species * oil * dosage (*F*=7,992, d.f. = 14; *P*<0.0001) were significant (Table 4).

The comparison of the relative toxicity of *C. sativa* and *H. lupulus* EOs by rmp analyses showed that *C. sativa* EO was more toxic than *H. lupulus* EO against *A. albopictus* and *P. acuta*, while *H. lupulus* was more toxic than *C. sativa* against *C. dipterum* (Fig. 1). In particular, *C. sativa* EO was able to kill 100% of *P. acuta* snails already from a concentration of 100 µL L^{-1}, while the same mortality was reached by *H. lupulus* EO only at 400 µL L^{-1}. On the contrary, while *H. lupulus* EO caused 100% of *C. dipterum* mortality starting from 400 µL L^{-1}, *C. sativa* EO at the same dosage killed 70% of *C. dipterum* nymphae (Tab. 4). Consistently, rmp analyses showed a significant different susceptibility among species to the EOs. In detail, for both the EOs the most sensitive species was *P. acuta* followed by *C. dipterum* while the less sensitive species was *A. albopictus* (Table 5).

4 Discussion

The chemical characterization of the ~~essential oil~~EOs is a crucial step before any kind of biological assay. ~~(Panizzi et al., 1993).~~The composition of the ~~essential oil~~EO of *C. sativa* is in good agreement with those reported in literature, with myrcene, *β*-caryophyllene, *α*-pinene, terpinolene and *α*-humulene as the main constituents (Bertoli et al., 2010;~~ Nissen et al., 2010;~~ Marchini et al., 2014; Nissen et al., 2010).

The composition of the ~~essential oil~~EO of *H. lupulus* is very dependent on the cultivar. In fact, the different cultivars are used to impart different properties to the beer (i.e. type of aroma, bitterness intensity, etc.). The composition of our ~~essential oil~~EO is the typical one of the American aroma variety Cascade, with high percentages of myrcene and *α*-humulene (Nance and Setzer, 2011). ~~The chemical characterization of the essential oil is a crucial step before any kind of biological assay (Panizzi et al., 1993).~~

Results showed a good toxic activity of *C. sativa* and *H. lupulus* EOs against the tested species. The effectiveness of the EOs even at low dosages highlighting their promising potential as control agents against the two problematic invasive species *A. albopictus* and *P. acuta*. Although hop and hemp are well-known aromatic and medicinal plants, moderate knowledge is available on their toxic effect ~~of Cannabaceae~~ on arthropods. However, our study is consistent with previous reports showing that aqueous extracts of *C. sativa* are able to repel or kill insects ~~and mites~~ (~~Bajpai and Sharma, 1992;~~ Jalees et al., 1993) and phytopathogenic nematodes ~~(*Heterodera cajani, Tylenchorhynchus brassicae, Hoplolaimus indicus, Rotylenchulus reniformis*)~~ (~~Haseeb et al., 1978;~~ Mojumder et al., 1989). Several studies also reported that *C. sativa* extracts exert fungicidal and bactericidal activities (~~Kaushal and Paul, 1989;~~ Upandhyaya and Gupta, 1990; Vijai et al., 1993). Besides, a recent study showed that *H. lupulus* EO exerts a strong repellent action against post-harvest grains insect pests (Bedini et al., 2015). To this regard, the chemical analyses showed that also the *C. sativa* EO contains high percentage of volatile compounds, such as ß-caryophyllene, caryophyllene oxide, limonene and myrcene that are powerful insect repellents (Bedini et al., 2015; Bougherra et al., 2015~~; Kashyap et al., 1991~~).

The results of toxicity tests against *A. albopictus* ~~With regard to *A. albopictus*, our results~~ are in line with previous researches showing the toxic effect of numerous plants ~~essential oils~~EOs against *A. albopictus* and other mosquitoes ~~(Benelli, 2015c)~~. For instance, the susceptibility of the Asian tiger mosquito larvae to the two Cannabaceae EOs resulted to be similar to that of~~to~~ *Achillea millefolium* EO (LC_{50}=211.3 ppm; Conti et al., 2010), *Azadirachta indica* (Meliaceae) EO (LC_{50} = 267.13; Benelli et al., 2015a) ~~and its fractions at different polarity (LC_{50} =142.28 to 209.73 ppm; Benelli et al., 2015a)~~, *Foeniculum vulgare* EO (LC_{50} = 142.9 ppm; Conti et al., 2010) and, to the EO extracted from fresh leaves of *Hyptis suaveolens* (Lamiaceae) (LC_{50} = 240.30 ppm; Conti et al., 2012a). On the contrary, *C. sativa* and *H. lupulus* EOs resulted less toxic against *A. albopictus* than EOs from other plants showing LC_{50} values under 100 ppm ~~EOs~~ such as the one from *Allium macrostemon* (Amarillidaceae) (LC_{50} =

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73); several Cupressaceae species with LC_{50} ranging from 37 (*Cupressus benthamii*) to 194 ppm; *Coleus aromaticus* and *Ocimum basilicum* (Lamiaceae) (LC_{50} = 76 and 11 ppm, respectively); several Rutaceae species ~~wild and cultivated plants of *Ruta chalepensis* (Rutaceae)~~ with (~~LC_{50}~~ values ranging from 35 (*Ruta chalepensis*) to 69 ppm (*Toddalia asiatica*~~.66 and 33.18 ppm, respectively; Conti et al., 2013)~~), ~~*Allium tuberosum* (LC_{50} = 17.90 ppm; Liu et al., 2015); *Eucalyptus urophylla* and *E. camaldulensis* (LC_{50} = 31 and 96 ppm, respectively; Cheng et al., 2009); *Toddalia asiatica* (LC_{50} = 69 ppm; Liu et al., 2013), *Clinopodium gracile* (LC_{50} = 43 ppm; Chen et al., 2013), *A. macrostemon* (LC_{50} = 73 ppm; Liu et al., 2014a), *Zanthoxylum avicennae* (LC_{50} = 49 ppm; Liu et al., 2014b).~~ (Pavela, 2015a, and reference therein).

In this work, acute toxicity varied considerably with the EO and the species tested. This is likely due to differences in the chemical composition of the two EOs. Such differen~~tce in the~~ EOs efficacy, however, could be due not only to the presence in the EOs of different bioactive ~~a different toxicity~~ chemical compounds (Bakkali et al., 2008) but also to their relationship as mixture~~of the EOs~~. In fact, the contribution of its singles compounds to the final EO's effect could be not only additional but also synergistic or anthagonistic (Hummelbrunner and Isman 2001, Pavela 2008, 2014). For instance, myrcene and α-pinene, two of the major chemical compounds of the *H. lupulus* and *C. sativa* EO, respectively, have been shown to have a clear antagonistic or synergistic effect against the Culicidae *Culex quinquefasciatus* larvae depending on the compound to which they were coupled (Pavela, 2015b). However, ~~but also to a different susceptibility of the *A. albopictus* populations of different geographical origin. Notably, with the exception of the *R. chalepensis* EO (Conti et al., 2013), EOs toxicity tests against tiger mosquitoes from Asian populations gave lower LC_{50} values respect to the ones performed with European mosquitoes strains.~~even if the toxicity of the hemp and hop EOs is lower than the one of some other aromatic plants, their use could be industrially highly convenient. Actually, from a practical point of view, the major problems that prevent the production on large scale

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of botanical insecticides (BIs) based on EOs are, along with their variability in qualitative and quantitative composition, the limited production and their high price (Pavela, 2015b). In this regards, both hemp and hops, because of their extensive cultivation and high EOs content could allow an economically convenient industrial production and an expansion of the utilization of BIs. Moreover, recently it has been showed that, at least in the case of hop, it is possible to obtain residual EO with excellent bioactive properties also from the brewery spent hops, showing that EOs utilization can add value also to by-product of the main industry (Bedini et al., 2015).

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Interestingly, t~~T~~he toxicity assays showed that both EOs are also effective in killing the invasive snail *P. acuta*. In particular, *C. sativa* EO resulted effective even at low dosages. Consistently, t~~T~~his freshwater snail has been ~~found susceptible to pesticides and industrial by-products (Bernot et al., 2005; Seeland et al., 2013) and~~ recently found to be susceptible also to the EOs of the ~~two~~ Mediterranean aromatic plants *Achillea millefolium* and *Haplophyllum tuberculatum* (Benelli et al., 2015b). ~~Beside~~Alongside EOs, also other aromatic plants extracts showed toxicity against freshwater snails with LC_{50} values similar to those recorded in our experiments. Recently, da Silva et al. (2013) reported molluscicidal activity of ground seeds of *Moringa oleifera* Lam. (Lamiales~~:~~ Moringaceae) against three species of snails, including *Physa marmorata* Guilding (LC_{50} = 339 ppm), an intermediate host of *Trichobilharzia* ~~(Pinto et al., 2015)~~ and *Echinostoma* ~~(Maldonado et al., 2001; Pinto and Melo, 2012)~~. Similarly, molluscicidal activity was reported for various compounds extracted from plants belonging to the Apocynaceae (Singh et al. 2005, 2010), Cupressaceae, Lauraceae, Myrtaceae, Pittosporaceae and Zingiberaceae (Singh and Singh, 2009; Teixeira et al., 2012), Lamiaceae, (Salama et al., 2012), Pinaceae (Lahlou, 2003) and Euphorbiaceae (Schall et al., 2001; Singh et al., 2010, 2005~~, 2010~~).

It is noteworthy that *C. sativa* EO resulted to be more toxic against the target species *P. acuta* respect to the non-target mayfly *C. dipterum*. Even if the use of plant-borne pesticides is recommended because reputed more safe for humans and the environment than synthetic pesticides, very little information is available on their side effects on non-target fauna. The available information indicates that they may exhibit toxicity also against components of the aquatic plankton (Conti et al., 2014; Duringer et al., 2010)~~such effects may vary widely depending on the species~~. Indeed, Conti et al. (2014) showed that the tea tree, *Melaleuca alternifolia* EO is more toxic to the non-target *Daphnia magna* Straus (Cladocera: Daphniidae) than against the target species *A. albopictus* (LC_{50} = 80.637 and 250 ppm, respectively). Nevertheless, the same EO resulted to have low toxicity against the brine shrimp *Artemia salina* L. (LC_{50} = 500 ppm ca) (McCage et al., 2002), and to be non-toxic for the rainbow trout *Oncorhynchus mykiss* (Walbaum) (Salmoniformidae~~:~~ Salmonidae) eggs (Marking et al., 1994). Such differences in the EOs toxicity among organisms could be due to their different metabolism. In particular, the toxic activity of the EOs could be based on the inhibition of the acetylcholinesterase activity. Actually, such inhibition has been shown by several plant extracts on insects (Ryan and Byrne, 1988) and by the monoterpene constituents of EOs (Mills et al., 2004). Another possible mechanism suggested to explain the fungicidal activity of ~~essential oils~~EOs may involve the disruption of the cell membrane affecting its permeability (Mukhtar et al., 2013). Also the time of application and the concentration could be used to trim the botanical pesticides in order to obtain a good activity against target organisms affecting as less as possible the non-target ones. For instance, recently it has been showed that EO toxicity against these non-target organisms may depend on the applied concentration and time of exposure. Pavela (2014) showed that short-term exposure of *D. magna* to *Pimpinella anisum* EO induce a significant rise in the fertility and thus exerted a positive effect on daphnia numbers.

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Such variability in the effectiveness and in the physiological action of EOs may allow the formulation of insecticides and molluscicides trimmed on the target species but it also strongly indicates the need of an assessment of their acute or chronic toxicity not only on the target but also on other non-target aquatic organisms. Moreover, since EOs have different effects such as reduced fertility, repellency and antifeedancy also at sub-lethal doses (Alzogaray et al. 2011; Giatropoulos et al., 2012; Hummelbrunner and Isman, 2001; Pavela, et al., 2007), further research could determine if a significant decrease in the population of target insects can be achieved at doses lower than the lethal ones therefore reducing the side effects on non-target organisms and on the environment.

~~Such variability in the effectiveness and in the physiological action of EOs may allow the formulation of insecticides and molluscicides trimmed on the target species but it also strongly indicates the need of an assessment of their acute or chronic toxicity not only on the target but also on other non-target aquatic organisms.~~

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5 Conclusions

This study contributes to the knowledge about the bioactivity of chemically characterized *C. sativa* and *H. lupulus* ~~essential oils~~EOs. Both the oils are able to exert a good toxic effect against the invasive disease vectors *A. albopictus* and *P. acuta*. The low-cost and large availability coupled with t~~T~~he much stronger effectiveness of the hemp ~~essential oil~~EO against the target snail over the non-target mayfly suggests that it could be a very promising tool for the development of new low-cost environmental friendly insecticides and molluscicides.

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Figure captions

Fig. 1. Comparison of the toxicity of *Cannabis sativa* and *Humulus lupulus* essential oils against *Aedes albopictus*, *Physella acuta* and *Cloeon dipterum*. Values < 1 indicate more toxicity of *C. sativa* respect to *H. lupulus* essential oil. Bars crossing the zero line indicate that the difference of effectiveness is not statistically significant. *A. albopictus*, white rectangle; *C. dipterum*, grey rectangle*;*, *P. acuta*, black rectangle.

Table 1. Chemical composition (%) of the *Cannabis sativa* and *Humulus lupulus* essential oils used in the toxicity assays

Constituents[a]	**LRI**[a]	**LRI**[b]	***C. sativa***	***H. lupulus***
Propyl butanoate	898	896		0.1±0.00
α-Pinene	941	939	7.7±0.29	0.2±0.06
Camphene	955	955	0.2±0.06	
Isopentyl propanoate	970			2.0±0.15
Sabinene	978	976	0.2±0.06	
β-Pinene	982	980	3.7±0.26	1.1±0.20
Myrcene	993	991	22.9±1.00	68.0±1.79
α-Phellandrene	1007	1006	0.3±0.15	
δ-3-Carene	1010	1011	0.6±0.20	
2-Methylbutyl isobutyrate	1015	1015 [c]		1.0±0.17
α-Terpinene	1020	1018	0.3±0.06	
Methyl heptanoate	1027	1028 [c]		0.5±0.10
p-Cymene	1028	1026	0.5±0.15	
Limonene	1033	1032	3.9±0.38	1.0±0.21
1,8-Cineole	1034	1033	0.2±0.00	
(*Z*)-*β*-Ocimene	1042	1040	0.7±0.15	
(*E*)-*β*-Ocimene	1053	1050	3.9±0.21	0.1±0.00
γ-Terpinene	1063	1062	0.3±0.06	
Methyl 6-methylheptanoate	1087	–		0.4±0.12
Terpinolene	1090	1088	12.0±0.90	
2-Nonanone	1092	1091		0.2±0.06
Linalool	1101	1098	0.3±0.00	0.6±0.10
Nonanal	1104	1102		0.2±0.00
Methyl octanoate	1128	1127 [c]		0.4±0.06
p-Cymen-8-ol	1185	1183	0.5±0.10	
α-Terpineol	1191	1189	0.2±0.06	
Methyl 4-nonenoate	1210	–		0.1±0.06
Methyl nonanoate	1228	1229		0.2±0.06
2-Undecanone	1292	1291		0.1±0.00
Carvacrol	1301	1298	0.2±0.00	
Methyl 4-decenoate	1311	1309 [d]		0.9±0.17
Methyl geranate	1325	1323		0.3±0.12
α-Copaene	1377	1376		0.1±0.00
Geranyl acetate	1383	1383		0.1±0.06
(*Z*)-Caryophyllene	1406	1404	0.7±0.15	
β-Caryophyllene	1419	1418	18.7±1.02	3.7±0.32
β-Copaene	1430	1430 [c]		0.2±0.00
trans-*α*-Bergamotene	1438	1439	1.5±0.35	
α-Humulene	1455	1454	6.2±0.32	13.3±0.95
(*E*)-*β*-Farnesene	1459	1458		0.3±0.15

9-*epi*-Caryophyllene	1468	1467	2.3±0.26	
γ-Muurolene	1478	1477	0.2±0.06	0.4±0.17
β-Selinene	1487	1485	1.6±0.17	0.2±0.06
α-Selinene	1495	1494	1.5±0.32	0.3±0.10
α-Muurolene	1500	1499		0.2±0.00
β-Bisabolene	1508	1509	0.4±0.17	
trans-*γ*-Cadinene	1514	1513	0.2±0.06	0.5±0.15
Geranyl isobutyrate	1516	1514		0.5±0.06
δ-Cadinene	1524	1524	0.2±0.06	0.7±0.20
Selina-3,7(11)-diene	1544	1542	0.6±0.17	
Germacrene B	1557	1556	0.2±0.00	
Caryophyllene oxide	1582	1581	3.7±0.42	0.3±0.10
Humulene oxide II	1607	1606	1.0±0.36	0.7±0.17
1-*epi*-Cubenol	1630	1627		0.1±0.00
T-Cadinol	1641	1640		0.2±0.00
α-Cadinol	1654	1653		0.2±0.06
Monoterpene hydrocarbons			57.2	70.4
Oxygenated monoterpenes			1.4	1.5
Sesquiterpene hydrocarbons			34.3	19.9
Oxygenated sesquiterpenes			4.7	1.5
Non-terpene derivatives			–	6.1
Total identified			97.6	99.4

[a], Chemical constituents ≥ 0.1%; LRI: [a] experimental linear retention index on DB-5 column; [b] from Adams (1995), except [c] from NIST Chemistry WebBook (http://webbook.nist.gov/chemistry/), [d] from Makkumrai et al. (2014)

Table 2. Toxicity of the essential oil (EO) of *Cannabis sativa* and *Humulus lupulus* against larvae of the targhet species *Aedes albopictus*, adults of *Phyta marmorata* and nymphs of the non-targhet species *Cleon dipterum*

	EO	LC_{50} [a]	LC_{90} [b]	Slope ± SE	Intercept ± SE	$\chi 2$ (df) [c]
A. albopictus	*C. sativa*	301.560	693.999	4.432 ± 0.511	-11.168 ± 1.073	**7.61** (3)
	H. lupulus	330.855	643.825	4.452 ± 0.481	-9.988 ± 1.283	**7.12** (3)
C. dipterus	*C. sativa*	282.174	631.961	3.660 ± 0.649	-8.968 ± 1.564	**1.26** (3)
	H. lupulus	219.787	391.121	5.120 ± 0.785	-11.991 ± 1.869	**2.59** (2)
P. marmorata	*C. sativa*	35.370	46.691	10.627 ± 1.207	-16.457 ± 1.915	**4.99** (2)
	H. lupulus	118.653	227.921	4.520 ± 0.788	-9.376 ± 1.628	**0.27** (1)

[a] Concentration of the extract that kills 50 % of the exposed organisms, [b] concentration of the extract that kills 90 % of the exposed organisms. Data are expressed as µL L^{-1}; [c] Chi-square; (df), degrees of freedom; [d] Values in bold indicate $P > 0.05$.

Table 3. Acute toxicity of *Cannabis sativa* and *Humulus lupulus* essential oils against the problematic invasive species *Aedes albopictus* (fourth instar larvae) and *Physella acuta* and the non target species *Cloeon dipterum*

Dosage ($\mu L\ L^{-1}$)	Mortality (% ± SE)					
	C. sativa			*H. lupulus*		
	A. albopictus	*C. dipterum*	*P. acuta*	*A. albopictus*	*C. dipterum*	*P. acuta*
0	0.00±0.00a	0.00±0.00a	0.00±0.00a	0.00±0.00a	0.00±0.00a	0.00±0.00a
25	0.00±0.00a	0.00±0.00a	3.33±1.67a	0.00±0.00a	0.00±0.00a	0.00±0.00a
50	1.67±1.67a	0.00±0.00a	90.00±10.00b	0.00±0.00a	0.00±0.00a	3.33±3.33a
100	3.33±3.33a	3.33±3.33a	100.00±0.00b	1.67±1.67a	6.67±3.33a	40.00±15.28b
200	25.00±5.00b	36.67±8.82b	100.00±0.00b	20.00±2.89b	36.67±3.33b	83.33±12.02c
300	41.67±8.33b	50.00±11.55b	100.00±0.00b	36.67±17.64bc	70.00±5.77c	93.33±6.67c
400	75.00±5.00c	70.00±15.28b	100.00±0.00b	55.00±2.89c	96.67±3.33d	100.00±0.00c
500	81.97±6.53c	80.00±11.55b	100.00±0.00b	88.33±4.41d	100.00±0.00d	100.00±0.00c

Each datum represents the mean of three replicates, each setup with 20 specimens (*A. albopictus* and *P. acuta*) or ten specimens (*C. dipterum*). Different letters indicate significant differences (GLM, Tukey's b post hoc test, $P < 0.05$).

Table 4. Relative susceptibilities of larvae of the target species *Aedes albopictus*, adults of *Physella acuta* and nymphs of the non-target species *Cloeon dipterum* to *Cannabis sativa* and *Humulus lupulus* essential oils (EOs)

EOs		*A. albopictus*	*P. acuta*
C. sativa	*P. acuta*	**8.868**[a]	
	C. dipterum	1.050[b]	**0.118**[c]
H. lupulus	*P. acuta*	**2.787**[a]	
	C. dipterum	**1.514**[b]	**0.543**[c]

Relative median potency analyses (rmp) values of the comparisons: [a], *A. albopictus vs P. acuta;* [b], *A. albopictus vs C. dipterum*; [c], *P. acuta* vs *C. dipterum*. Values < 1 indicates more susceptibility; Values > 1 indicates less suscettibility. Bold indicates significant values (95% CI ≠ 1).

Fig. 1