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ORIGINAL PAPER

Mediterranean essential oils as effective weapons against the West Nile vector *Culex pipiens* and the *Echinostoma* intermediate host *Physella acuta*: what happens around? An acute toxicity survey on non-target mayflies

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Abstract Mosquitoes (Diptera: Culicidae) represent a threat for millions of people worldwide, since they act as vectors for important pathogens, including malaria, yellow fever, dengue and West Nile. Second to malaria as the world's most widespread parasitic disease, infection by trematodes is a devastating public health problem. In this study, we proposed two essential oils from plants cultivated in Mediterranean regions as effective chemicals against mosquitoes and freshwater snails vectors of *Echinostoma* trematodes. Chemical composition of essential oils from *Achillea millefolium* (Asteraceae) and *Haplophyllum tuberculatum* (Rutaceae) was investigated. Acute toxicity was evaluated against larvae of the West Nile vector *Culex pipiens* (Diptera: Culicidae) and the invasive freshwater snail *Physella acuta* (Mollusca: Physidae), an important intermediate host of many parasites, including *Echinostoma revolutum* (Echinostomidae). Acute toxicity of essential oils was assessed also on a non-target aquatic organism, the mayfly *Cloeon dipterum* (Ephemeroptera: Baetidae). *Achillea millefolium* and *H. tuberculatum* essentials oils were mainly composed by oxygenated monoterpenes (59.3 and 71.0 % of the whole oil, respectively). Chrysanthenone and borneol were the two major constituents of *Achillea millefolium* essential oil (24.1 and 14.2 %, respectively). Major compounds of *H. tuberculatum* essential oil were *cis-p*-menth-2-en-1-ol and *trans-p*-menth-2-en-1-ol (22.9 and 16.1 %, respectively). In acute toxicity assays, *C. pipiens* LC_{50} was 154.190 and 175.268 ppm for *Achillea millefolium* and *H. tuberculatum*, respectively. *P. acuta* LC_{50} was 112.911 and 73.695 ppm for *Achillea millefolium* and *H. tuberculatum*, respectively, while the same values were 198.116 and 280.265 ppm for *C. dipterum*. Relative median potency analysis showed that both tested essential oils were more toxic to *P. acuta* over *C. dipterum*. This research adds knowledge on plant-borne chemicals toxic against invertebrates of medical importance, allowing us to propose the tested oils as effective candidates to develop newer and safer vector control tools.

Keywords *Achillea millefolium* · *Haplophyllum tuberculatum* · GC-MS analysis · Mosquito-borne diseases · Echinostomiasis · Trematoda · Non-target aquatic organisms

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Introduction

Mosquitoes (Diptera: Culicidae) represent a key threat for millions of people worldwide, since they act as vectors for important pathogens, including malaria, yellow fever, dengue, West Nile and chikungunya (Jensen and Mehlhorn 2009; Mehlhorn 2011), and parasites, such as filariasis (Benedict et al. 2007; Paupy et al. 2009; Caminade et al. 2012). Mosquito's bite causes also local skin reactions, as well as serious allergic and systemic responses such as angiodema and urticaria (Peng et al. 1999). In this scenario, vector control is a crucial prevention tool. Culicidae larvae are usually targeted using organophosphates and insect growth regulators. Indoor residual spraying and insecticide-treated bed nets are also employed to reduce transmission of malaria in tropical countries. These chemicals have negative effects on human health and/or the environment and induce resistance in a number of mosquito species (Severini et al. 1993; Hemingway and Ranson 2000; Sun et al. 2011; Lees et al. 2014).

On this basis, eco-friendly tools have been recently implemented to enhance control of mosquito vectors. Renewed interest has been devoted to the potential of sterile insect technique (SIT) for suppression of mosquito vectors, with special reference to the genus *Anopheles* (Lees et al. 2014; Oliva et al. 2014; see also Benelli 2015). Furthermore, huge efforts have been carried out investigating the efficacy of plant-borne products (see Azizullah et al. 2014; Benelli et al. 2015 for recent reviews), with special reference to plant essential oils and extracts, against Culicidae. Many compounds have been reported as good toxics against mosquitoes, acting as adulticidal (Govindarajan and Sivakumar 2012), larvicidal (Amer and Mehlhorn 2006a; Michaelakis et al. 2009a; Pavela 2009; Benelli et al. 2013; Giatropoulos et al. 2013), ovicidal (Govindarajan et al. 2011), oviposition deterrents (Benelli et al. 2014b, c), growth and/or reproduction inhibitors (Rajkumar and Jebanesan 2005; Pushpanathan et al. 2006), and/or adult repellents (Amer and Mehlhorn 2006b; Gleiser et al. 2011; Koliopoulos et al. 2010; Conti et al. 2012a, b, 2013).

Second to malaria as the world's most widespread parasitic disease, infection by trematodes is a devastating public health problem (Chitsulo et al. 2000; Keiser and Utzinger 2004; Singh et al. 2010). Besides schistosomiasis, also food-borne trematode infections, caused by liver flukes (*Clonorchis*, *Fasciola*, *Opisthorchis*), lung flukes (*Paragonimus*) and intestinal flukes (*Echinostoma, Fasciolopsis,* heterophyids), are a key health issues worldwide, with special reference to Southeast Asia and Western Pacific. Globally, more than 56 million people are infected and 750 million people live in endemic areas (Keiser and Utzinger 2004; WHO 2014). The most common tool against infection by trematodes is chemotherapy, based on orally administered drugs in infected humans. However, this approach often leads to incomplete elimination of the infection, high costs and possible drug resistance (Singh et al. 2010; Sohn et al. 2011; da Silva et al. 2013). A better way to tackle the problem is to destroy snails that act as intermediate hosts of Trematoda. Niclosamide is the only commercially available molluscicide recommended by the World Health Organisation for large-scale use in control programs against Trematoda. However, from an ecotoxicological point of view, this chemical is highly toxic against non-target soft-bodied aquatic organisms and leads to marked decline of them in several areas (da Silva et al. 2013, but see Andrews et al. 1982 for non-toxic effects on humans). To overcome this challenge, some eco-friendly tools have been developed, including the use of plant-borne compounds as molluscicides to control freshwater snails (Chifundera et al. 1993; Brackenbury 1999; Lahlou 2003; Radwan et al. 2008; Jaiswal and Singh 2009; Kumar et al. 2010; Rapado et al. 2011; da Silva et al. 2013; Teixeira et al. 2012; Singh et al. 2010).

On this basis, we proposed two essential oils from plants cultivated in different regions of the Mediterranean basin as potential sources of compounds effective against mosquitoes, and freshwater snails acting as vectors of Trematoda. Our research investigates the chemical composition of essential oils from *Achillea millefolium* L. (Asterales: Asteraceae) and *Haplophyllum tuberculatum* (Forssk.) A. Juss. (Sapindales: Rutaceae). Acute toxicity of the two essential oils was evaluated against larvae of the West Nile vector *Culex pipiens* (L.) (Diptera: Culicidae) (Fig. 1a) and the invasive freshwater snail *Physella acuta* (Draparnaud) (Mollusca: Physidae) (Fig. 1b), an important intermediate host for many parasites (Toledo et al. 1999, 2000; Faltýnková 2005; Faltýnková and Haas 2006; Muñoz-Antoli et al. 2006, 2008; Barragán-Sáenz et al. 2009; Kraus et al. 2014), including the worldwide distributed trematode *Echinostoma revolutum* Looss (Echinostomida: Echinostomidae) and the nematode *Parastrongylus cantonensis* (Chen) (Rhabditida: Angiostrongylidae) (Hai et al. 2009). The acute toxicity of *A. millefolium* and *H. tuberculatum* was also assessed on a non-target aquatic organism sharing the same ecological niche of *C. pipiens* larvae and *P. acuta* snails, the nymphs of the mayfly *Cloeon dipterum* L. (Ephemeroptera: Baetidae) (Fig. 1c).

Materials and methods

Plant species

The aerial parts of *H. tuberculatum* and *A. millefolium* were collected in June 2013 from M'sila (35° 42′ 07.4″ N, 4° 32′ 49.7″ E) and Jijel (36° 49′ N, 05° 46′ E), respectively. They were identified by one of the authors (H.L.). Plant voucher specimens were deposited at the laboratory of Phytotherapy Applied to Chronic Diseases, University Setif, Algeria.

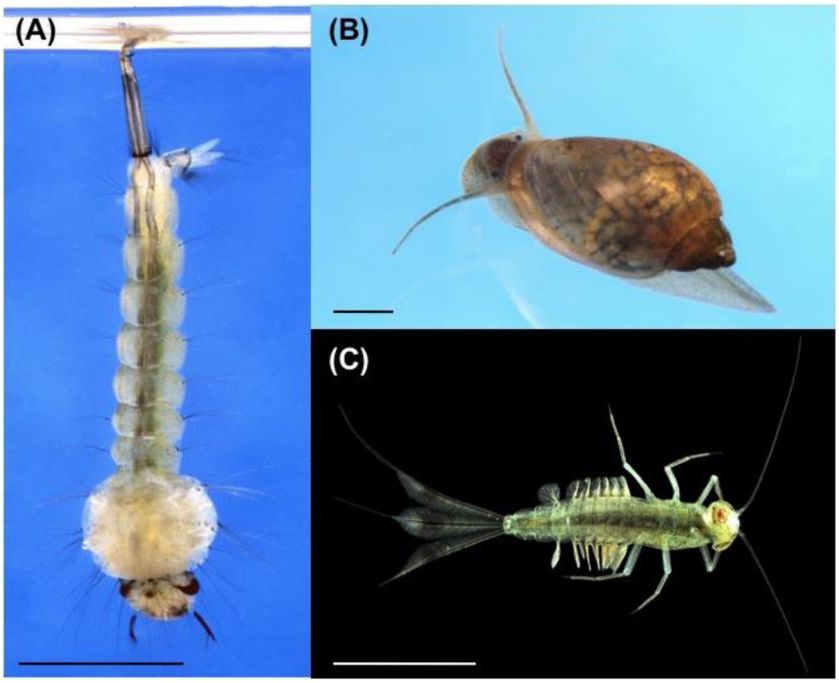

Fig. 1 An overview of the three aquatic invertebrates tested in this study: **a** larvae of the West Nile vector *Culex pipiens* (L.) (Diptera: Culicidae), **b** the *Echinostoma* intermediate host *Physella acuta* (Draparnaud) (Mollusca: Physidae) and **c** the non-target mayfly *Cloeon dipterum* L. (Ephemeroptera: Baetidae). The scale is 1 mm

Essential oil extraction and GC-MS analysis

Achillea millefolium and *H. tuberculatum* aerial parts were hydro-distilled in a Clevenger-type apparatus for 4 h. Yield in essential oil was 0.25 % (*w*/*w*) for *A. millefolium* and 0.10 % for *H. tuberculatum*. Gas chromatography (GC) analyses were carried out with an HP-5890 Series II instruments equipped with HP-WAX and HP-5 capillary columns (30 m× 0.25 mm, 0.25-μm film thickness), working with this temperature program: 60 °C for 10 min, ramp of 5 °C/min up to 220 °C; injector and detector temperatures 250 °C; carrier gas was helium (2 mL/min); detector dual FID; split ratio 1:30; injection of 0.5 μL (10 % hexane solution). Components identification was carried out, for both columns, by comparing their retention times with those of pure authentic samples and by means of their linear retention index (lri), relative to the series of *n*-hydrocarbons.

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. Constituents identification was based on comparison of retention times with those of authentic samples; this implied comparing 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) (Stenhagen et al. 1974; Massada 1976; Jennings and Shibamoto 1980; Swigar and Silverstein 1981; Davies 1990; Adams 1995). Molecular weights of all identified substances were confirmed by gas chromatography-chemical ionisation mass spectrometry (GC-CIMS), using methanol as ionising gas.

Culex pipiens rearing

Culex pipiens tested in the experiments originated from Pisa (Italy) and were collected from field water tanks at the Department of Agriculture, Food and Environment from August to October 2014. *Culex pipiens* were reared in laboratory conditions (24±1 °C; 50±5 % RH, natural photoperiod). Young larvae were isolated in glass tube (20 larvae/tube) with tap water and a small amount of cat food until they reached the fourth instar. Only newly emerged fourth instar larvae were used for bioassays (Conti et al. 2014).

Physella acuta rearing

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 characterisation (see below). *Physella 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 (*Lactuca sativa* L.) were used as foodstuff. Only adult snails were used for bioassays.

Molecular characterisation of *Physella acuta*

Physella acuta was identified at specific level with molecular characterisation. Genomic DNA was isolated from whole animals using standard phenol/chloroform methods. Two (one nuclear and one mitochondrial) genes were amplified by PCR. The nuclear small subunit ribosomal RNA (SSU-rRNA or 18S) gene sequence was determined using the universal eukaryotic forward primer 18S F9 5′-CTGGTTGATCCTGCCAG-3′ (Medlin et al. 1988) and the 18S R1513 Hypo reverse primer 5′-TGATCCTTCYGCAGGTTC-3′ (Fokin et al. 2008). The mitochondrial DNA sequence was obtained for a segment of the mitochondrial cytochrome c oxidase subunit I (COI), using the forward primer LCO1490 5′-GGTCAACAAATCATAAAGATATTGG-3′ and the reverse primer LCO2198 5′-TAAACTTCAGGGTGACCAAAAAATCA-3′ (Folmer et al. 1994). The PCR amplifications were performed by adding aliquots (100 ng) of purified DNA to 50-μL reaction mixtures containing 2 mM $MgCl_2$, 250 mM of dNTP, 1 U of Taq DNA polymerase (Polymed, Florence, Italy) and 0.2 mM of each primer. Reactions were accomplished using a GeneAmp PCR System 2400 (Applied Biosystems, Foster City, CA, USA) that, in the case of the nuclear gene (18S), was programmed with an amplification profile of 30 cycles of 30 s at 94 °C, 30 s at 55 °C and 120 s at 72 °C, followed by 5 min at 72 °C for final extension. The amplification regime used for the mitochondrial gene (COI) was in agreement to Wethington and Lydeard (2007). PCR products were purified using Quantum Prep PCR Kleen Spin columns (Bio-Rad, Hercules, CA, USA) and sequenced in both directions with an ABI Prism 310 automated DNA sequencer (Applied Biosystems). To establish the taxonomic assignment of the snail used in this study, a comparative analysis of its sequences to those homologous recorded in GenBank/EMBL databases was carried out using a BLAST search. The 18S and COI gene sequences determined in this study are available from the GenBank/EMBL databases under the accession numbers KP171533 and KP171534, respectively.

Cloeon dipterum rearing

Cloeon dipterum nymphs were collected from field water tanks at the Department of Agriculture, Food and Environment in September and October 2014, identified at specific level following the keys reported in Grandi (1960), then reared in laboratory conditions (24±1 °C; 50±5 % R.H.; natural early autumn photoperiod) in 5-L beakers containing mineral water and fed with a small amount of leaf litter and cat food. Late instars nymphs (length 3.9 mm±0.2 m) were used for bioassays.

Larvicidal activity of essential oils against *Culex pipiens*

Three groups of 20 larvae (fourth instar) were isolated in 250-mL beakers and exposed for 24 h to dosages of 10, 25, 50, 80, 100, 200, 300 and 400 ppm of *A. millefolium* and *H. tuberculatum* essential oils. 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 (WHO 1981; Benelli et al. 2013, 2014a, b; Conti et al. 2013). Mortality percentage rates were corrected using Abbott's formula (Abbott 1925).

Acute toxicity of essential oils against *Physella acuta*

Three groups of 20 specimens of *P. acuta* were isolated in 250-mL beakers and exposed for 24 h to dosages of 10, 25, 50, 80, 100, 200, 300 and 400 ppm of *A. millefolium* and *H. tuberculatum* essential oils in tap water containing 0.025 % of Tween 80. The beakers were covered with chiffon to prevent snails from falling out. None of the snails were fed during this period. At the end of the exposure period, mortality was checked. Control experiments were executed similarly and simultaneously as the treatments. Two hundred fifty-millilitre 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). *Physella acuta* mortalities were reported as an average of three replicates, data were also used to calculate the LC_{50} value. Since no mortality was observed in the control treatment, the mortality percentage rates were not corrected.

Acute toxicity of essential oils against 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 of 10, 25, 50, 80, 100, 200, 300 and 400 ppm of *A. millefolium* and *H. tuberculatum* essential oils in tap water containing 0.025 % of Tween 80. Two hundred fifty-millilitre 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 (Conti et al. 2014). *Cloeon dipterum* mortalities were reported as an average of three replicates, data were also used to calculate the LC_{50} value. Since no mortality was

observed in the control treatment, the mortality percentage rates were not corrected.

Data analysis

Mortality data of *C.pipiens*, *P. acuta* and *C. dipterum* were transformed into arcsine/proportion values before statistical analysis. Data were processed with JMP, using a general linear model (GLM) with three factors, the tested invertebrate, the essential oil and the dosage: $y_j = \mu + TI_j + EO_j + D_j + TI_j * EO_j + EO_j * D_j + TI * D_j + TI_j * EO_j * D_j + e_j$, in which y_j is the observation, μ is the overall mean, TI_j is the tested invertebrate (j=1–3), EO_j is the tested essential oil (j=1–2), D_j is the dosage (j=1–9), $TI_j * EO_j$ is the interaction invertebrate * essential oil, $EO_j * D_j$ is the interaction essential oil * dosage, $TI_j * D_j$ is the interaction invertebrate * dosage, $TI_j * EO_j * D_j$ is the interaction invertebrate * essential oil * dosage and e_j is the residual error. Averages were separated by Tukey's HSD test. $P<0.05$ was used for the significance of differences between means.

Median lethal dose (LD_{50}) against the three tested species was calculated by Log-probit regressions by the SPSS 22.0 software (SPSS Inc., Chicago, IL, USA). Significant differences between LD_{50} values were determined by estimation of confidence intervals of the relative median potency. Differences among LD_{50} values were judged as statistically significant when values in the 95 % confidence interval of relative median potency analyses were≠1.0.

Results

Chemical composition of essential oils

GC-MS analyses on the essential oils obtained from the aerial parts of *A. millefolium* and *H. tuberculatum* led to the identification of 35 and 39 compounds, representing 96.1 and 91.3 % of the whole oil, respectively (Table 1). *Achillea millefolium* and *H. tuberculatum* essentials oils were mainly composed by oxygenated monoterpenes (59.3 and 71.0 % of the whole oil, respectively) (Table 2). Chrysanthenone and borneol were the two major constituents of *A. millefolium* essential oil (24.1 and 14.2 % of the whole oil, respectively). The major compounds of *H. tuberculatum* essential oil were *cis*-*p*-menth-2-en-1-ol and *trans*-*p*-menth-2-en-1-ol (22.9 and 16.1 %, respectively) (Table 1).

Molecular characterisation of *Physella acuta*

The PCR amplification products of the 18S and COI genes of the freshwater snail tested in this study produced a 1.80-kb and a 0.65-kb DNA band, respectively. Sequencing of the 18S

Table 1 Chemical composition (%) of the essential oils from *Achillea millefolium* and *Haplophyllum tuberculatum* aerial parts used in the acute toxicity assays

Constituents	l.r.i.	*Achillea millefolium*	*Haplophyllum tuberculatum*
(*E*)-2-Hexenal	856	–	0.5
(*Z*)-4-Heptenal	900	–	0.4
α-Pinene	941	1.8	3.2
Camphene	955	3.1	1.3
Sabinene	978	0.7	0.1
β-Pinene	982	–	1.4
2,3-Dehydro-1,8-cineole	992	–	0.7
Mesitylene	996	0.7	–
δ-3-Carene	1010	–	1.0
α-Terpinene	1020	1.6	1.2
p-Cymene	1028	4.3	1.3
β-Phellandrene	1033	–	4.6
1,8-Cineole	1034	3.0	–
Santolina alcohol	1039	0.9	–
(*Z*)-β-Ocimene	1042	–	0.2
(*E*)-β-Ocimene	1053	–	0.1
γ-Terpinene	1063	2.9	0.1
cis-Sabinene hydrate	1070	0.3	–
1-Octanol	1072	–	2.1
Terpinolene	1090	1.4	0.1
2-Nonanone	1092	–	0.2
Linalool	1101	0.3	1.1
2,6-Dimethylphenol	1106	9.0	–
cis-*p*-Menth-2-en-1-ol	1122	0.5	22.9
Chrysanthenone	1126	24.1	–
trans-*p*-Menth-2-en-1-ol	1142	0.6	16.1
Camphor	1145	4.4	–
cis-Chrysanthenol	1164	0.6	–
Borneol	1168	14.2	0.6
Ethyl benzoate	1171	–	0.3
4-Terpineol	1178	8.2	0.4
p-Cymen-8-ol	1185	–	0.2
α-Terpineol	1191	1.0	0.2
cis-Piperitol	1194	–	6.4
trans-Piperitol	1206	–	9.8
1-Octyl acetate	1213	–	0.9
Isobornyl formate	1233	–	0.6
Piperitone	1254	–	3.5
1-Decanol	1273	–	0.3
Isobornyl acetate	1286	0.6	6.4
Thymol	1292	0.6	–
2-Undecanone	1292	–	0.4
Carvacrol	1301	–	–
cis-Piperitol acetate	1332	–	1.9
Neryl acetate	1366	–	0.2
(*E*)-Geranylacetone	1454	–	0.1

Table 1 (continued)

Constituents	l.r.i.	*Achillea millefolium*	*Haplophyllum tuberculatum*
ar-Curcumene	1483	–	0.2
Germacrene D	1482	1.0	–
(*E*)-β-Ionone	1485	–	0.1
β-Selinene	1487	0.3	–
β-Sesquiphellandrene	1523	–	0.2
δ-Cadinene	1524	0.3	–
Ledol	1566	0.3	–
Spathulenol	1577	0.5	–
Caryophyllene oxide	1582	0.4	–
Viridiflorol	1591	2.6	–
10-*epi*-γ-Eudesmol	1623	0.7	–
1-*epi*-Cubenol	1629	0.4	–
T-Cadinol	1641	0.3	–
Himachalol	1649	0.5	–
β-Eudesmol	1650	4.0	–
Total identified		96.1	91.3

l.r.i. linear retention index

and COI genes provided a 1805 base pairs (bp) and a 655-bp DNA sequence, respectively, with a GC content of 51.69 and 33.44 %, respectively. Comparative analysis of the sequenced genes to those homologous recorded in GenBank/EMBL databases provided, for both genetic markers analysed, the maximum value of sequence identity (100 %) with the species *P. acuta*.

Acute toxicity experiments

Achillea millefolium and *H. tuberculatum* essential oils were toxic against all tested aquatic organisms (Table 3). GLM showed no significant differences between essential oils (F=0.555, $d.f.$ = 1; P=0.458), whereas a significant effect of the tested invertebrate species (F=59.464, $d.f.$ = 2; P<0.0001) and essential oil dosage (F=226.341, $d.f.$ = 8; P<0.0001) was

Table 2 Principal chemical classes (%) in the essential oils from aerial parts of *Achillea millefolium* and *Haplophyllum tuberculatum*

Main chemical classes	*Achillea millefolium*	*Haplophyllum tuberculatum*
Monoterpene hydrocarbons	15.8	14.6
Oxygenated monoterpenes	59.3	71.0
Sesquiterpene hydrocarbons	1.6	0.4
Oxygenated sesquiterpenes	9.7	–
Apocarotenoids	–	0.2
Non-terpene derivatives	9.7	5.1
Total identified	96.1	91.3

Table 3 Acute toxicity of essential oils from *Achillea millefolium* and *Haplophyllum tuberculatum* aerial parts against the West Nile vector *Culex pipiens* (fourth instar larvae), the freshwater snail *Physella acuta* and the non-target mayfly *Cloeon dipterum*

Invertebrate	Essential oil	Dosage (ppm)	Mortality (%±SE)
Culex pipiens	*Achillea millefolium*	Control	3.14±3.14 jk
		10	32.09±6.52 efghij
		25	10.69±10.69 ijk
		50	13.84±6.92 hijk
		80	15.10±3.29 ghijk
		100	11.95±6.19 hijk
		200	62.26±13.21 bcdef
		300	88.68±6.54 abc
		400	96.23±3.77 a
	Haplophyllum tuberculatum	Control	0 k
		10	3.77±0.00 ijk
		25	14.47±14.47 ijk
		50	10.063±6.00 hijk
		80	9.43±3.26 hijk
		100	7.55±3.77 hijk
		200	41.51±6.80 defghi
		300	86.79±4.99 abcd
		400	100 a
Physella acuta	*Achillea millefolium*	Control	0 k
		10	0 k
		25	0 k
		50	0 k
		80	21.67±6.67 fghijk
		100	25.00±10.41 fghijk
		200	98.33±1.67 a
		300	100 a
		400	100 a
	Haplophyllum tuberculatum	Control	0 k
		10	0 k
		25	0 k
		50	25.00±10.41 fghijk
		80	50.00±5.774 cdefgh
		100	80.00±5.00 abcde
		200	96.67±3.33 a
		300	100 a
		400	100 a
Cloeon dipterum	*Achillea millefolium*	Control	0 k
		10	0 k
		25	0 k
		50	0 k
		80	0 k
		100	16.67±6.67 fghijk
		200	60.00±10.00 bcdefg
		300	83.33±3.33 abcd
		400	96.67±3.33 a

Table 3 (continued)

Invertebrate	Essential oil	Dosage (ppm)	Mortality (%±SE)
	Haplophyllum tuberculatum	Control	0 k
		10	3.33±3.33 jk
		25	0 k
		50	0 k
		80	3.33±3.33 jk
		100	6.66±3.33 ijk
		200	13.33±6.67 hijk
		300	43.33±6.67 defghi
		400	93.33±6.67 ab

Each datum represents the mean of three replicates, each setup with 20 specimens (*C. pipiens* and *P. acuta*) or ten specimens (*C. dipterum*). Different letters indicate significant differences (general linear model, Tukey's HSD test, $P<0.05$)

found. In addition, the interactions of invertebrate * oil ($F=12.520$, *d.f.* = 2; $P<0.0001$), oil*dosage ($F=3.910$, *d.f.* = 8; $P=0.0004$), invertebrate * dosage ($F=10.377$, *d.f.* = 2; $P<0.0001$) and invertebrate * oil * dosage ($F=2.529$, *d.f.* = 16; $P=0.0025$) were significant (Table 3).

Concerning *C. pipiens*, LC_{50} was 154.190 and 175.268 μL/L for *A. millefolium* and *H. tuberculatum*, respectively. In *P. acuta* acute toxicity trials, LC_{50} ranged from 112.911 and 73.695 ppm for *A. millefolium* and *H. tuberculatum*, respectively, while, for *C. dipterum,* LC_{50} values were 198.116 and 280.265 ppm (Table 4). Relative median potency analysis of probits confirmed a significantly different susceptibility among species as shown by GLM outcomes. *Physella acuta* resulted as the most susceptible of the tested species to both *A. millefolium* and *H. tuberculatum* essential oil while the non-target species *C. dipterum* as the less susceptible one (Table 5). With regard to the two essential oils, relative median potency analysis showed that the *A. millefolium* essential oil was more toxic than the *H. tuberculatum* one for *C. dipterum* and *C. pipiens*, while *H. tuberculatum* essential oil was found more toxic than *A. millefolium* essential oil for *P. acuta* (Fig. 2).

Table 5 Relative median potency analysis comparing toxicity of essential oils from *Achillea millefolium* and *Haplophyllum tuberculatum* against *Culex pipiens* larvae, the freshwater snail *Physella acuta* and the non-target mayfly *Cloeon dipterum*

Essential oil	Invertebrate	*Cloeon dipterum*	*Physella acuta*
Achillea millefolium	*Culex pipiens*	**1.252**	**0.723**
	Physella acuta	**1.733**	–
Haplophyllum tuberculatum	*Culex pipiens*	**1.610**	**0.416**
	Physella acuta	**3.866**	–

Values>1 indicates less susceptibility; values<1 indicates more susceptibility. Significant differences between values are marked in bold (95 % CI≠1)

Discussion

GC-MS analyses led to the identification of 35 and 39 compounds in *A. millefolium* and *H. tuberculatum* essential oils, respectively. Both oils were mainly composed by oxygenated monoterpenes. Chrysanthenone and borneol were the two major constituents of *A. millefolium* essential oil (24.1 and 14.2 % of the whole oil, respectively). Borneol has been also found as a major component (7.1 %) in *A. millefolium* oil extracted from plants collected in Italy, even if in this latter oil, eucalyptol (14.2 %) and β-pinene (12.4 %) have been reported as the most abundant compounds (Conti et al. 2010). *H. tuberculatum* major constituents were *cis-p*-menth-2-en-1-ol and *trans-p*-menth-2-en-1-ol, and this is at variance with previous analyses on *H. tuberculatum* oils

Table 4 Toxicity of essential oils from *Achillea millefolium* and *Haplophyllum tuberculatum* against *Culex pipiens* larvae, the freshwater snail *Physella acuta* and the non-target mayfly *Cloeon dipterum*

Invertebrate	Essential oil	LC_{50}[a]	95 % CI[b]	Slope±SE	Intercept±SE	χ^2 (*df*)[c]
Culex pipiens	*Achillea millefolium*	154.190	135.484–171.820	4.320±0.481	−9.453±1.102	**0.19**[d] (2)
	Haplophyllum tuberculatum	175.268	74.958–267.516	4.452±0.481	−9.988±1.114	**5.49** (2)
Physella acuta	*Achillea millefolium*	112.911	105.011–123.237	7.408±0.877	−15.207±1.755	**6.86** (5)
	Haplophyllum tuberculatum	73.695	67.172–80.605	4.769±0.500	−8.906±0.945	**4.32** (5)
Cloeon dipterum	*Achillea millefolium*	198.116	179.249–223.468	7.225±1.205	−16.595±2.728	**2.39** (5)
	Haplophyllum tuberculatum	280.265	215.092–386.387	4.764±0.845	−11.660±2.056	**8.86** (5)

[a] Lethal concentration (LC) killing 50 % of exposed specimens. Data expressed as ppm

[b] Confidence interval

[c] Chi-squared degrees of freedom

[d] Values in bold indicate $P>0.05$

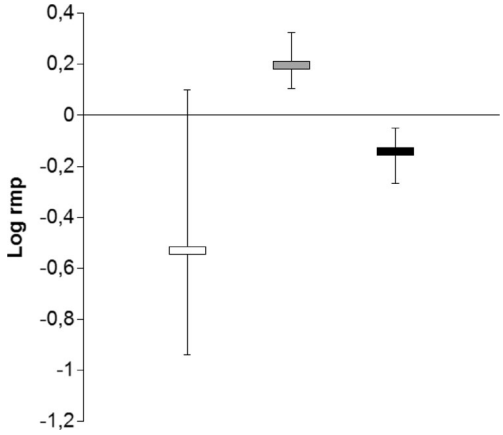

Fig. 2 Relative median potency (*rmp*) analysis comparing the toxicity of the essential oils (EOs) of *Achillea millefolium* and *Haplophyllum tuberculatum* against the mosquito *Culex pipiens*, the freshwater snail *Physella acuta* and the non-target mayfly *Cleon dipterum*. Negative values indicate that *A. millefolium* EO is more toxic than *H. tuberculatum*. T-bars indicate confidence intervals. T-bars crossing zero indicate no differences in effectiveness. *C. pipiens* (white), *P. acuta* (grey) and *C. dipterum* (black)

extracted from plants with a different geographical origin (e.g., Oman), where β-phellandrene (23.3 %), limonene (12.6 %), *(Z)*-β-ocimene (12.3 %), β-caryophyllene (11.6 %), myrcene (11.3 %) and α-phellandrene (10.9 %) have been reported as the most abundant components (Al-Burtamani et al. 2005). In *H. tuberculatum* essential oil from Iran, the main constituents were limonene (27.3 %) and α-pinene (21.9 %) (Yari et al. 2000). These differences may be a function of the plant origin and cultivation, as already observed for different plant species (Tchoumbougang et al. 2005; Noudjou et al. 2007; Conti et al. 2013, 2014).

Results from toxicity experiments testing *A. millefolium* and *H. tuberculatum* against larvae of *Culex pipiens* showed that both essential oils are able to exert good toxicity rates against this mosquito species, also when tested at low dosages. Insecticidal activity of *H. tuberculatum* essential oil against *Culex quinquefasciatus* Say (concentration-dependent mortality of larvae, pupae and adults at dosages ranging from 50 to 2000 ppm) has been reported by Mohsen et al. (1989), while *Achillea millefolium* essential oil has been previously proved as effective at low dosages against the Asian tiger mosquito, *Aedes albopictus* (Skuse) (LC_{50}=211.3 ppm) (Conti et al. 2010). The present research broadens the number of low-cost essential oils able to exert toxicity against larval instars of the West Nile vector *Culex pipiens*. Concerning plant species growing in Mediterranean regions, good examples include the larvicidal activity showed by essential oils from Egyptian Lamiaceae, such as *Thymus capitatus* Hoff. and Link., and *Marrubium vulgare* L. (LC_{50}=100 and 200 ppm, respectively) (Salama et al. 2012), as well as by essential oils from Greek species belonging to the *Satureja* genus (Lamiaceae) (LC_{50} ranging from 37.7 to 64.4 ppm) (Michaelakis et al. 2008) and *Mentha, Melissa* and *Salvia* (LC_{50} ranging from 47.88 to 140.42 ppm) (Koliopoulos et al. 2010). Similarly, essential oils extracted by Greek plants of *Dianthus caryophyllus* L. (Caryophyllaceae), *Lepidium sativum* L. (Cruciferae), *Illicium verum* Hook. f. (Illiaceae) and *Pimpinella anisum* L. (Umbelliferae) all showed LC_{50} ranging from 15.24 to 68.62 ppm (Kimbaris et al. 2012). Also, essential oils from Greek *Citrus* species, including sweet orange (*Citrus sinensis* L.), lemon (*Citrus limon* L.) and bitter orange (*Citrus aurantium* L.), are effective toxics against *Culex pipiens* larvae (LC_{50} were 51.50, 30.14 and 39.81 ppm, respectively) (Michaelakis et al. 2009b), and similar results have been achieved also testing Apiaceae from the same geographical region (LC_{50} ranging from 40.26 to 96.96 ppm) (Evergetis et al. 2009).

Toxic assays conducted on the snail *P. acuta* highlighted that the tested essential oils are effective toxics, even at low dosages. This freshwater snail has been found susceptible to pesticides and industrial by-products (Bernot et al. 2005; Seeland et al. 2013). However, to the best of our knowledge, this is the first report about toxicity of essential oils against this intermediate host of Trematoda. Recently, da Silva et al. (2013) reported molluscicidal activity of the ground seed of *Moringa oleifera* Lam. (Lamiales: Moringaceae) against three species of snails, including *Physa marmorata* Guilding (LC_{50}=0.339 g/L), an intermediate host of *Trichobilharzia* (Pinto et al. 2014) and *Echinostoma* (Maldonado et al. 2001; Pinto and de Melo 2012). More generally, a number of plant-borne molluscicides are effective against freshwater snails at dosages comparable to those tested in our experiments (i.e. from <1 to 200 ppm ca), with special reference to several groups of compounds from plants, mainly belonging to the Apocynaceae family (e.g. *Cascabela thevetia* (L.) Lippold and *Alstonia scholaris* L. R. Br.) (Singh et al. 2005, 2010), some Azorean, Cupressaceae, Lauraceae, Myrtaceae, Pittosporaceae and Zingiberaceae (Singh and Singh 2009; Teixeira et al. 2012), Mediterranean Lamiaceae, such as *T. capitatus* and *M. vulgare* (Salama et al. 2012), some Moroccan species from the genus *Pinus* (Pinaceae) (Lahlou 2003), and the *Euphorbia* genus (Euphorbiaceae) (Schall et al. 2001; Singh et al. 2005, 2010), even if this latter require careful handling due to toxic properties to humans and aquatic organisms (Clark et al. 1997).

Acute toxicity experiments conducted on mayflies, *Cloeon dipterum*, showed lower toxicity of the tested essential oils, if compared to toxicity exerted against the target species *P. acuta*. This finding is noteworthy, since it allows us to candidate the above-mentioned natural products as molluscicides to be employed at low doses with limited impacts on non-target aquatic fauna. By contrast, other essential oils showed high toxicity against non-target aquatic organisms. A good example is the acute toxicity exerted by tea tree,

Melaleuca alternifolia (Maiden & Betche) Cheel (Myrtaceae), essential oil against *Daphnia magna* Straus (Cladocera: Daphniidae), even at low dosages (LC_{50}=80.637 ppm) (Conti et al. 2014). However, the same essential oil was not so toxic against other crustacean species, such as the brine shrimp *Artemia salina* L. (LC_{50}=500 ppm ca) (McCage et al. 2002), and was found non-toxic for rainbow trout eggs [*Oncorhynchus mykiss* (Walbaum) (Salmoniformidae: Salmonidae)] (Marking et al. 1994). However, further research is needed to investigate chronic and/or reproductive toxicity of the tested essential oils both non-target aquatic aquatic organisms, as well as to shed light on how these natural compounds exert toxicity against snails and arthropods; with special reference to the latter, we believe that the toxic activity exerted by tested essential oils could be due to the anticholinesterase activity of monoterpene constituents (Mills et al. 2004).

Overall, this research adds knowledge about the chemical composition and bioactivity of the essential oils extracted from *A. millefolium* and *H. turbeculatum*. The tested oils are able to exert good toxicity rates against the West Nile vector *C. pipiens* and the invasive freshwater snail *P. acuta*, an intermediate host of *Echinostoma* trematodes. Both essential oils are less toxic against the non-target mayfly *C. dipterum* over targeted snails, allowing us to propose these natural products as suitable candidates for the development of newer and safer molluscicides.

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Authors' contributions GB and BC conceived and designed the experiments. GB, GF, SB, FC, PLC, GDG and BC performed the experiments. GB, SB, GF and GDG analysed the data. GB, GF, SB, SA, FB, HL, GDG and BC contributed reagents/materials/analysis tools. GB wrote the paper.

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