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Molluscicidal activity of four Apiaceae essential oils against the freshwater snail *Radix peregra*

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Abstract Snails of the family Lymnaeidae are an essential link in the transmission of zoonotic diseases. *Radix peregra* is a European freshwater snail and a susceptible intermediate host of *Fasciola hepatica*, the causing agent of fascioliasis. Essential oils (EOs) extracted from *Anethum graveolens* (dill), *Cuminum cyminum* (cumin), *Foeniculum vulgare* var. *vulgare* (bitter fennel) and *Petroselinum crispum* (plain leaf parsley) were characterized by GC and GC–MS. Seven EOs and 11 constituents were first screened through a single-dose bioassay against *R. peregra* (10 mg L^{-1} for juveniles and 50 mg L^{-1} for egg masses and mature snails). EOs from parsley, cumin and bitter fennel (leaves plus stems) were highly active towards eggs and adults at 50 mg L^{-1}. Subsequently, dose and time–lethality bioassays were performed against adults to determine lethal parameters ($LC_{50;90}$ and $LT_{50;90}$). Estimated 48 h LC_{50s} varied from 13.7 to 46.5 mg L^{-1}, with *P. crispum* fruits EO exhibiting the most significant activity. EOs from cumin fruits and bitter fennel infrutescences, and cuminaldehyde, were the most time-effective treatments when assessed by continuous exposure (LT_{50} for a 50 mg L^{-1} dose = 15.1, 19.3 and 19.5 h, respectively). A short-time exposure (8 h) to bitter fennel EOs was effective for the control of adults ($LT_{50} \leq 25$ h). The present study uncovers the potential of four well-known Apiaceae species as natural sources of biomolluscicides.

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Key message

- Plant species of the Apiaceae family remain undervalued with regard to their activity as biomolluscicides.
- Bitter fennel, parsley, cumin and dill EOs are highly toxic against the freshwater snail *Radix peregra*, an alternative intermediate host of fascioliasis.
- Estimated 48 h LC_{50} of the several EOs and compounds tested range from 13.7 to 46.5 mg L^{-1}.
- Parsley fruit EO exhibits the most significant dose effectiveness of the assayed EOs.
- A short-time exposure (8 h) to two bitter fennel EOs was effective for snails' control (LT_{50} of 23.0 and 25.7 h) in laboratory conditions.

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- EOs and compounds from the four studied Apiaceae have great potential as biomolluscicides.

Introduction

Snails of the family Lymnaeidae are freshwater pulmonate snails (Gastropoda: Pulmonata) that can inhabit a variety of wet and aquatic habitats, from large lakes to small temporary ponds (Watson and Dallwitz 2005). As intermediate hosts of plant-borne trematodes, lymnaeids species play a key role in the transmission of fascioliasis and others trematodiases of major medical and veterinary relevance. Fascioliasis, which is caused by species of the genus *Fasciola* L. (Digenea: Fasciolidae), is recognized as a major veterinary problem of vertebrate domestic livestock, causing a decrease in animals' productivity and considerable economic losses (Correa et al. 2010).

Although the natural and primary intermediate host of *Fasciola hepatica* L. in Europe is *Galba* (sin. *Lymnaea*) *truncatula* Müller (Bargues et al. 2001), others lymnaeids from European populations have been identified as alternative intermediate hosts of this fasciolid under special natural conditions (Dreyfuss et al. 2000; Bargues et al. 2001). Climate changes and abnormal environmental phenomena may greatly influence host–parasite interactions, and by consequence the epidemiological patterns of fascioliasis and other trematodiases (Mas-Coma et al. 2009). The spreading power of fascioliasis is also related to the remarkable adaptive abilities of fasciolids to colonize new environments and to infect different intermediate and definitive hosts species all over the world (Mas-Coma et al. 2005). For instance, young specimens of *Radix* (sin. *Lymnaea*) *peregra* Müller (Lymnaeidae) were found to be susceptible to *F. hepatica* infection in laboratory conditions (Sindou et al. 1991; Dreyfuss et al. 1997). The prevalence of this species in some habitats where the *G. truncatula* is less abundant, and the occurrence of cattle fasciolosis in some of these areas, gives additional evidences of its potential as an alternative or secondary intermediate of the common liver flukes (Relf et al. 2009). In the Azorean island of S. Miguel, the density of *R. peregra* is effectively increasing in some locations where *G. truncatula* populations were previously found (Teixeira et al. 2011), including areas with identified cases of fascioliasis (Martins 1991). *R. peregra* may also display a role in the transmission of the giant liver fluke *Fascioloides magna* Bassi in cervids and domestic ruminants (Faltynkova et al. 2006), being also a facultative host of *F. gigantica* in near east, middle east and southern states of old USSR (Bargues et al. 2001).

As all plant-borne trematodes, including flukes, require aquatic intermediate hosts to accomplish part of their life cycle, the elimination of this essential link is the most effective way of preventing transmission of fascioliasis and others trematodiases (WHO 1995; Singh et al. 2010). The drainage of water bodies and/or treatment with synthetic molluscicides (i.e. niclosamide, sodium pentachlorophenate, copper sulphate, etc.) are possible measures stated by the World Health Organization for the control of these intermediate hosts (WHO 1995). Niclosamide is recommended to be used in management programs (WHO 1993); however, its toxicity to non-target organisms (from highly to very highly toxic to freshwater fishes) (USEPA 2013) rises some concerns. In order to mitigate the impact of molluscicides application on aquatic systems and organisms, several efforts have been developed to discover molluscicidal products of natural sources that could present lower risks (Singh et al. 2010). In that context, and considering the long history of uses, effectiveness, low cost and reduced toxicity, plants have been considered as ideal and sustainable sources of bioactive chemicals to control snails' populations (WHO 1983; Singh et al. 1996). Plants species belonging to the Euphorbiaceae, Asteraceae, Leguminosae, Phytolaccaceae, Apocynaceae and Solanaceae families have been frequently assessed for this purpose (WHO 1983; Singh et al. 2005, 2010). The activity of EO or their constituents towards schistosomiasis and fascioliasis intermediate hosts, and pest snails have been confirmed in several studies (Lahlou and Berrada 2001; Kumar and Singh 2006; Jaiswal and Singh 2009; Mc Donnell et al. 2016). Nevertheless, Apiaceae (Umbelliferae) species remain undervalued with regard to their potential as freshwater snails' control agents, despite their large spectrum of activities, namely against insect species (Yeom et al. 2012; Evergetis et al. 2013; Kim et al. 2013; Sousa et al. 2013).Thus far, extracts of *Trachyspermum ammi* (L.) Sprague, *Ferula assafoetida* L. and *Carum carvi* L. were formerly tested against the liver flukes' natural host in Southern Asia, *Lymnaea acuminata* Lamark (Singh et al. 1997; Kumar and Singh 2006). However, to the extent of our knowledge, scientific reports concerning the potential of Apiaceae EOs for the control of trematode intermediate hosts are by far scarcest.

Hence, we herein propose to evaluate the molluscicidal potential of EOs and compounds from four well-known and worldwide cultivated Apiaceae species [*Anethum graveolens* L., *Cuminum cyminum* L., *Foeniculum vulgare* Mill. and *Petroselinum crispum* (Mill.) Nym ex A.W. Hill]. For this purpose, we performed screening assays against several developmental stages (eggs, juveniles and adults) of *Radix peregra*, a facultative intermediate host of trematodes in Europe, including *Fasciola hepatica*. Furthermore,

dose- and time-related activities of EOs/compounds on the adult form of this freshwater snail were also investigated.

Materials and methods

Plant material and extraction of essential oils

Foeniculum vulgare subsp. *vulgare* var. *vulgare* (bitter fennel) culture was started from seeds collected from plant of a wild-growing population in north of Portugal (41°36′03.8″N, 8°26′50.25″W), and *A. graveolens* (dill) and *P. crispum* var. *neapolitanum* (plain leaf parsley) cultures were established from commercial seeds (packed by N.V. Somers, Co.). *F. vulgare* var. *vulgare* was identified based on both morphological and chemical characterizations, and voucher specimens (stems with leaves and inflorescences and fruits) are deposited at the University of Porto (Portugal) herbarium (accession number PO1000MFF). All plants were grown in an open-air experimental field. Fresh stems and leaves were collected from dill plants during the fruit pre-ripening stage and from bitter fennel plants during the vegetative phase. Green infrutescences bearing fully formed fruits were harvested from dill, parsley and bitter fennel plants, after 5, 8 and 14 months of growth, respectively. A total of 150–300 g of the aforementioned fresh samples was separately submitted to a 2-h hydrodistillation with 2–3 L of deionized water in a Clevenger-modified apparatus. EOs were collected and dried over anhydrous sodium sulphate before their storage in brown sealed vials at −20 °C. In addition, certified EOs from *P. crispum* (parsley) and *C. cyminum* (cumin) fruits were purchased from Sigma-Aldrich, Co (St. Louis, MO, USA) and included in the study.

Chemical analyses of essential oils

The quantitative and qualitative characterization of EOs was achieved by GC and GC–MS analyses as previously described (Sousa et al. 2013).

Chemicals

The following pure standard compounds that were identified in the EOs were included in bioassays: *trans*-anethole (99%), (S)-(+)-carvone (96%), cuminaldehyde (98%), estragole (98%), (+)-fenchone (99.5%), γ-terpinene (≥97%), (−)-β-pinene (99%), (−)-α-pinene and (+)-α-pinene (98%) were purchased from Sigma-Aldrich and Fluka (Aldrich chemical Co., St. Louis, MO, USA). The compounds myristicin (≥95%) and apiole (≥82%) were separated from parsley fruit EO by performing successive fractionations through gravity column chromatography as described in Sousa et al. (2013). The compound $CuSO_4$ (pro-analysis grade, purity 99%, purchased from Merck) was included in assessments as a control molluscicide. Solvents (*n*-hexane, ethyl acetate and ethanol) were of reagent grade (≥99.9%).

Preparation of EOs/compounds emulsions

EOs/compounds were initially diluted in pure ethanol (300 mg mL^{-1}). The dispersion of the oil fraction into a small volume of spring water was achieved using an ultrasonic homogenizer (Sonopuls HD 2200 from BANDELIN) coupled with a 3-mm microtip (amplitude 302 μm/ss; power 25–30%; time 5 s on–off cycles for 2 min) to obtain stock emulsions (33 mg EO per mL^{-1}). Different volumes were pipetted, according to the doses to be tested, and vigorously mixed with spring water making up the final volumes used in bioassays (10 mL for eggs and juveniles and 220 mL for adults). Emulsions were left to stabilize 15 min before use. The spring water used in the preparation of emulsions is from S. Miguel Island (Povoação, Furnas, São Miguel Island, Azores Archipelago, Portugal) and commercialized under the trade name of "Gloria Patri" (water pH at 19 °C:6.95; total mineral content of 213 mg L^{-1}; HCO$_3^-$:81 mg L^{-1}; SiO$_2$:57 mg L^{-1}, Na$^+$:26 mg L^{-1}; Cl$^-$:14 mg L^{-1}; K$^+$:11.4 mg L^{-1}; NO$_3^-$:7.8 mg L^{-1}; SO4^{2-}:5.6 mg L^{-1}; Ca^{2+}:4.9 mg L^{-1}; Mg$^+$:4.6 mg L^{-1}).

Activity against *Radix peregra*

Animals

Radix peregra adult snails (with average shell length of 1.34 ± 0.20 cm) were caught in small freshwater bodies nearby livestock water tanks found in the *Povoação* locality (Southwest of São Miguel Island, Azores Archipelago, Portugal). Species identification has been confirmed by a malacologist, Professor Frias Martins A., and specimens (accession number DBUA-MT1533) are deposited in the molluscs' collection of the Biology Department of the University of the Azores (Ponta Delgada, São Miguel, Portugal). Animals were maintained in a polyethylene aquarium (35.7 cm × 23.5 cm × 13.4 cm) containing 3 L of spring water permanently aerated with air pumps and fed with lettuce leaves (*Lactuca sativa* L.). Snails were acclimatized under laboratory conditions (natural photoperiod and 23 ± 1 °C) for at least 72 h before being used in bioassays. In order to prevent any contamination, the aquarium was cleaned three times a week by removing excrements and dead snails and by renewing the water. Sexually mature snails were kept in the aquarium for reproduction purpose, to obtain egg masses.

Freshly laid egg masses (0–48 h old) were observed under the stereoscopic microscope to confirm their viability and ascertain their initial stage of embryonic development (4–8 cells) prior to the ovicidal assays. In addition, several egg masses were maintained under constant artificial aeration in a separated container until hatching. Juvenile snails of 48–72 h old were selected for the molluscicidal assays.

Ovicidal activity

R. peregra egg masses with less than 48 h were exposed to single-dose treatments of EOs/compounds (50 mg L^{-1} of spring water) to assess their ovicidal activity. Masses containing around 32 ($\pm$9.3) viable snail embryos were placed in freshly prepared emulsions (10 mL). Two sets of negative controls were included and performed simultaneously, one with spring water and the other with an ethanol aqueous solution in which the ethanol concentration was equivalent to that used in EOs/compounds' emulsions tested (0.1 µL mL^{-1}). Each treatment included at least six replicates (six-well plates). Egg masses were incubated under controlled temperature (23 $\pm$ 1 °C) and relative humidity (70 $\pm$ 5%) and exposed to natural photoperiod. Eggs were daily inspected to monitor their development. The ovicidal activity of the treatments was evaluate by calculating the percentage of hatching (snail emerged from the capsule) 15 days later (time for which the hatching in the control groups was $\geq$95%). Embryos were considered dead whenever cells became disaggregated and/or if they remained unhatched at the end of the experiment.

Single-dose screening assays against juveniles and adults

EOs/compounds' molluscicidal activity was assessed following the WHO guidelines for the immersion method (WHO 1983). A single-dose screening was performed with juveniles and adults using freshly prepared emulsions at 10 and 50 mg L^{-1} of spring water, respectively. Groups of ten juveniles were gently placed in six-well plates (ten per wells) with the help of a micropipette and a large tip. After removing the water, 10 mL of emulsions (10 mg L^{-1}) was carefully dispensed in each well. Concerning the immersion of the adults, groups of ten snails were transferred into glass Petri dishes and distributed randomly by disposable plastic beakers containing 220 mL of EOs/compounds emulsions (at 50 mg L^{-1} of spring water). To prevent adults from falling out or escape, cups were covered with a perforated plastic film or a Petri dish. A positive control with copper sulphate was included in both assays at the same concentration used for EOs/compounds. Again, two sets of negative controls, one with spring water and the other with ethanol aqueous solution (0.02 mL L^{-1} for juveniles and 0.1 mL L^{-1} for adults), were performed

simultaneously. Four to six replicates of ten snails per EOs/compounds/controls were included in each single-dose assay (40 $\leq n \leq$ 60). Snails' mortality was first determined after 24 h of exposure. The surviving snails were rinsed with spring water to remove emulsion residues, transferred into new cups containing an identical volume of spring water and fed with fresh lettuce leaves. Following a 24-h recovery period, the percentage of dead juveniles and adults was determined once again. The mortality was monitored by observation with a stereo microscope. Juveniles' death was confirmed by absence of heartbeat, whereas adults were considered dead when the deterioration of the tissues was evident (foot discoloration and body fluid leakage) or if they failed to react when prodded (typical withdrawal movements). Experiments were carried out under natural photoperiod at 23 $\pm$ 1 °C and 70 $\pm$ 5% of RH.

Dose–response assay against adults (LCs)

Based on the information obtained from the single-dose assay, we established to determine the LC values of the most lethal treatments (whenever adults' mortality >80%, after the recovery period). Besides, the compound (S)-(+)-carvone was also considered for further evaluations despite the first indication of its lower molluscicidal activity revealed in the preliminary assay. Owing to several reasons (high availability, lower cost and higher solubility in water) this oxygenated monoterpene exhibits great applicability in the context of plant-based biopesticides. Moreover, its bioactive potential has been shown and discussed in our previous work (Sousa et al. 2013).

The selected EOs and compounds were tested in the range of 10–60 mg L^{-1}, following the procedures described in the previous sections and the WHO guidelines. The positive control (CuSO$_4$) was tested at concentrations ranging from 0.1 to 1 mg L^{-1}. To establish a dose–response relationship, four to six replicates of ten snails per concentration and at least four concentrations per EO/compound were used (160 $\leq n \leq$ 300). The mortality percentage was recorded at 24 h of exposure and after the 24 h of recovery period to determine the concentrations causing 50 and 90% of lethality (LC$_{50}$ and LC$_{90}$).

Time–response assay against adults (LTs)

The evaluation of time-dependent lethality was performed with the most active EOs/compounds and (S)-(+)-carvone following the methodology above described. With this assay we assessed treatments' lethality as a function of times of exposure, in order to ascertain whether or not treatments' effectiveness was affected by the duration of exposure and in what extent. The concentration of

50 mg L^{-1} was chosen based on its proximity to most of the estimated LC_{90} values determined after the 24-h recovery period. Following the same criteria, the $CuSO_4$ (positive control) was assessed at 0.75 mg L^{-1}.

The time–lethality was assessed by continuous and short-term exposure (8 and 16 h). Adult snails that were exposed to treatments during 8 and 16 h were rinsed with spring water and left to recover in clean beakers containing fresh water and lettuce leaves. Subsequently, the mortality was monitored every 2 h or every hour if acuter effects were noticed. Snails submitted in continuation to treatments were monitored every 2 h, and the mortality percentage recorded up to 90% of death. In each assay, four replicates of ten individuals ($n = 40$) were used per treatment. For all three experiments, time started counting at the beginning of the exposure.

Statistical analysis

Data obtained in the single-dose assays (hatching and mortality percentages) were transformed by the arcsine function prior to the one-way analysis of variance (one-way ANOVA). Means of treatments were separated at 5% significance level by LSD test, available on IBM SPSS statistic package (version 20.0 for Windows, IBM corp., USA, 2011). Mortality percentages recorded in dose- and time-dependent experiments were corrected with the Abbott's formula (Abbott 1925) whenever mortality in the negative control exceeded 5%. LC_{50} and LC_{90} after 48 h (24 h of exposure more 24 h of recovery) were determined by probit analysis according to Litchfield and Wilcoxon (1949), using IBM SPSS statistic package. The same linear model was applied to time–response data, to determine EOs/compounds LT_{50} and LT_{90} (times required to cause 50 and 90% of adults' mortality) at the dose of 50 mg L^{-1}. Pearson Chi-squared (χ^2) test was used to assess the goodness of fit. A good fit of data to the model was found whenever χ^2 values were lower than χ^2 critical values for $n-2$ degrees of freedom, at 5% of significance level. Treatments' bioactivities were considered significantly different whenever the 95% confidence intervals of lethal concentrations or lethal times failed to overlap.

Results

Essential oils composition

The chemical characterization of bitter fennel, dill, parsley and cumin EOs used in the bioassays is presented in Table 1. The total number of compounds with relative content above 0.05% was variable among EOs (9–24), depending on the plant species, as well as on the plant

organ. The overall identification achieved for EOs constituents was about 98.5–99.9%.

EOs obtained from different organs of *F. vulgare* var. *vulgare* presented a similar qualitative composition, although with substantial differences in their compound contents. Fv-L + S accumulated high contents of several monoterpenes hydrocarbons (pinenes and phellandrenes) and lower contents of phenylpropene derivatives (30%), whereas Fv-I was richer in oxygenated compounds (65% estragole and 16% of fenchone). EOs of *A. graveolens* followed the same general trend although with some several qualitative differences. A large fraction of Ag-I (67%) was constituted by (*S*)-carvone, contrarily to Ag-L + S where this compound was not detected. Dill ether (3,9-epoxy-1-p-menthene) was detected in both dill EOs from green freshly collected materials (Ag-I and Ag-L + S), although with different percentages (22% in leaves + stems and 1.3% in infrutescences). Furthermore, α-phellandrene and limonene mixed with β-phellandrene represented, together, a significant part in both dill EOs (70 and 28% in leave + stems and infrutescences, respectively), roughly the double of their corresponding sum in bitter fennel EOs. Some qualitative and quantitative differences were also found in parsley EOs. Both presented phenylpropene derivatives (myristicin and apiole) as major compounds and high contents of pinenes (total of 30 and 44% of the EO). Nevertheless, the amount of the hydrocarbon monoterpenic fraction was superior in Pc-I (total of 72%), while the phenylpropene derivatives was prevailing in Pc-F (59.4%). The cumin fruit EO (Cc-F) was mainly composed by cuminaldehyde (39%) plus several monoterpenes hydrocarbons accounting for 48% of the EO. Despite the difference of their qualitative profiles profile, Cc-F and Pc-F presented similar proportion of oxygenated/non-oxygenated compounds.

Ovicidal and molluscicidal activity

The lethal activities recorded for the seven EOs and eleven volatile compounds tested against *R. peregra* eggs, juveniles and adult are presented in Fig. 1a–c. In general, EOs from the four Apiaceae species and their major compounds displayed distinct toxicities against the embryonic and post-embryonic stages of *R. peregra* when tested at unique dosage. The ovicidal activities recorded for treatments were highly variable (Fig. 1a) and significantly distinct ($F = 49.378$, *d.f.* = 133, $p = 0.000$) (Online Resource 1). In particular, Pc-F, (−)-β-pinene, Cc-F, *S*-(+)-carvone, (−)-α-pinene, myristicin, (+)-α-pinene, cuminaldehyde, Pc-I and Fv-L + S (cited by decreasing order of activity) exhibited the most interesting hatching inhibitory effects (mean hatching values ≤25%). Nonetheless, only the first two treatments showed comparable activity to that of the positive control. Satisfactory or moderate ovicidal

Table 1 Composition of essential oils (EOs) isolated from infrutescences (-I) stems and leaves (-L + S) and/or fruits (-F) of *Anethum graveolens* (Ag), *Cuminum cyminum* (Cc), *Foeniculum vulgare* var. *vulgare* (Fv) and *Petroselinum crispum* (Pc)

Compounds[a]	KI[b] RTx-5	KI ref.	Relative content (%)[c]						
			Dill		Cumin	Bitter fennel[g]		Parsley	
			Ag-I	Ag-L + S	Cc-F	Fv-I	Fv-L + S	Pc-I	Pc-F
α-Thujene[d,e]	927	931	tr	0. 1	0.2	tr	tr	tr	–
α-Pinene[d,e]	941	939	0.1	1.6	0.8	3.8	18.0	23.4	16.2
Sabinene[d,e]	978	975	tr	tr	–	1.0	2.9	–	–
β-Pinene[d,e]	984	980	–	–	12.4	–	–	20.8	13.6
Myrcene[d,e]	989	990	0.1	–	0.4	1.2	2.9	1.8	0.7
α-Phellandrene[d,e]	1000	1002	5.3	60.7	0.8	4.9	24.3	0.6	0.2
α-Terpinene[d,e]	1016	1017	0.1	4.0	0.1	0.2	2.6	–	tr
p-Cymene[d,e]	1026	1026	–	–	10.4	–	–	–	6.6
β- Phellandrene[d,e]	1035	1035	24.7	9.6	–	5.5	10.1	10.6	
Limonene[d]		1030			–	–	–	–	–
(Z)-β-Ocimene[d,e]	1039	1040	–	tr	–	–	0.1	0. 1	tr
(E)-β-Ocimene[d,e]	1051	1050	tr	–	–	–	0.3	tr	–
γ-Terpinene[d,e]	1064	1062	–	tr	15.8	0.2	–	0.2	0.3
1-Isopropenyl-4-methylbenzene[d,e]	1087	1088	–	–	–	–	–	–	0.6
Terpinolene[d,e]	1086	1088	–	0.1	0.7	–	–	–	–
2,5-Dimethyl styrene[d,e]	1097	1099	–	–		–	–	2.4	–
Fenchone[d,e,f]	1096	1087	–	–	–	15.7	6.2	–	–
1,3,8-p-Menthatriene[d,e]	1110	1114	tr	–	–	–	–	10.6	0.1
Fenchol[d,e]	1116	1117	–	–	–	–	0.1	–	–
cis-p-Mentha-2-en-1-ol[d,e]	1120	1122	–	0. 1	–	–	–	0.9	–
allo-Ocimene[d,e]	1128	1129	–	–	–	0.1	2.0	0.3	–
1,3,5,5-Tetramethyl-1,3-cyclohexadiene[e]	1130	…	–	–	–	–	–	–	0.7
trans-Pinocarveol[d,e]	1139	1139	–	–	0.2	–	–	–	–
Camphor[d,e]	1142	1146	–	–	–	0.1	–	–	–
Terpinen-4-ol[d,e]	1176	1177	–	–	0.2	0.1	–	0.1	–
Dill ether[d,e]	1182	1184	1.3	21.9	–	–	–	–	–
p-Cymen-8-ol[e]	1183	1183	–	–	–	–	–	0.2	0.1
α-Terpineol[d,e]	1184	1189	–	–	1.5	–	–	0.1	–
Dihydrocarveol[d,e]	1191	1192	0.3	tr	–	–	–	–	–
Myrtenal[d,e]	1192	1195	–	–	–	–	–	0.3	0.2
Estragole[d,e,f]	1199	1198	–	–	–	64.9	28.4	–	–
trans-Dihydrocarvone[d,e]	1198	1201	1.2	–	–	–	–	–	–
Cuminaldehyde[d,e,f]	1249	1239	–	–	39.4	–	–	–	–
(S)-Carvone[d,e,f]	1255	1242	66.4	tr	–	–	–	–	–
Phellandral[d,e]	1275	1276	–	–	0.3	–	–	–	0.1
p-Mentha-1,4-dien-7al[d,e]	1289	1278	–	–	9.7	–	–	–	–
trans-Anethole[d,e,f]	1290	1287	–	–	–	2.2	1.5	–	–
p-Mentha-1,3-dien-7al[d,e]	1292	1287	–	–	1.7	–	–	–	–
Carvacrol[d,e]	1300	1298	–	–	0.3	–	–	–	–
α-Copaene[d,e]	1373	1376	–	–	0.1	–	–	0.1	–
1,1,6-Trimethyl-1,2-dihydronaphthalene[e]	1377	–	–	–	0.5	–	–	–	–
β-Elemene[d,e]	1383	1388	–	–	0.1	–	–	0.6	0.1
E-Caryophyllene[d,e]	1417	1417	–	–	0.3	–	–	tr	0.1

Table 1 continued

Compounds[a]	KI[b] RTx-5	KI ref.	Relative content (%)[c]						
			Dill		Cumin	Bitter fennel[g]		Parsley	
			Ag-I	Ag-L + S	Cc-F	Fv-I	Fv-L + S	Pc-I	Pc-F
γ- Elemene[d,e]	1427	1430	–	–	–	–	–	0.1	–
E-β-Farnesene[d,e]	1454	1458	–	–	0.4	–	–	–	0.2
γ-Muurolene[d,e]	1466	1477	–	–	–	–	–	tr	0.1
Acoradiene[d,e]	1471	1471	–	–	2.3	–	–	–	–
γ- Gurjunene[d,e]	1475	1476	–	–	–	–	–	tr	0.1
Myristicin[d,e,f]	1537	1525	–	tr	–	–	–	–	31.5
Elemicin[d,e]	1567	1556	–	tr	–	–	–	–	3.4
Carotol[d,e]	1597	1600	–	–	0.2	–	–	–	8.6
1-Allyl-2,3,4,5-tetramethoxybenzene[d,e]	1607	1609	–	–	–	–	–	1.1	
Apiole[d,e]	1692	1687	–	0.1	–	–	–	24.3	15.9
Classes of compounds									
Monoterpenes hydrocarbons	30.3	76.1	41.6	16.9	63.2	71.9	39.1		
Oxygen-containing monoterpenes	69.2	22.1	53.3	15.9	6.3	1.6	0.4		
Sesquiterpenes hydrocarbons	0.0	0.0	3.7	0.0	0.0	0.8	0. 6		
Oxygen-containing sesquiterpenes	0.0	0.0	0.2	0.0	0.0	0.0	[h]		
Phenylpropene derivatives	0.0	0.1	0.0	67.1	29.9	25.4	59.4		
Total of identified content % (±SD)			99.5 (0.10)	98.5 (0.70)	98.8 (0.24)	99.9 (0.06)	99.5 (0.03)	98.9 (0.05)	99.5 (0.24)

tr—compounds were found in trace amount, below 0.05%; – not found

[a] Compounds listed by order of elution in a non-polar capillary column Rtx-5 (fused silica capillary column coated with 5% phenyl methyl polysiloxane)

[b] Kovats retention indices (KI) were determined by co-injection of samples with a series of *n*-alkanes in the Rtx-5 column

[c] Mean relative contents were determined for three or more injections

[d] Identification based on comparison of the calculated KI with those found in the database http://webbook.nist.gov/ and reported in the literature for equivalent capillary columns

[e] Identification based on mass spectra (MS) comparison

[f] Comparison of MS and KI with those of authentic standard injected separately

[g] Sousa et al. (2015b)

[h] One oxygen-containing sesquiterpene, carotol, was identified in the sample but as co-eluted product along with a phenylpropene derivative

activities were attributed to the treatments Ag-I, (+)-fenchone, apiole, *trans*-anethole, Ag-L + S and estragole, for which the hatching ranged from 37 to 71%.

Moreover, the newly emerged juveniles and adult snails showed different susceptibility to EOs and compounds (Fig. 1b, c). At the end of the experiments (48 h), mortalities varied from 3 to 59% ($F = 42.624$, $d.f. = 102$, $p = 0.000$) and 3 to 100% ($F = 42.318$, $d.f. = 97$, $p = 0.000$) for juveniles and adults, respectively (Fig. 1b, c) (Online Resource 1). We have observed moderate to noteworthy increases in lethality for the majority of compounds against juveniles and adults, respectively, during the recovery period (compare 24- and 48-h values in Fig. 1b, c). EOs from dill, parsley, cumin and bitter fennel (L + S), as well as the compound apiole, were the most

active treatments against juveniles at 24 h. Cuminaldehyde attained a similar activity against juveniles after the recovery period. Consistently, these treatments were highly effective against adults. In addition, Fv-I and the compounds *trans*-anethole, estragole, γ-terpinene and (−)-β-pinene exhibited a satisfactory activity against adults (>70%).

Dose–response

The toxicity of seven EOs and six individual compounds against adults' snails was dose dependent, which allowed the estimation of lethal concentrations within acceptable confidence limits (CLs) (Table 2). In general, most of the data fitted well in the assumptions of the linear model.

Fig. 1 a–c Lethal activity of *Anethum graveolens* (Ag), *Cuminum cyminum* (Cc), *Foeniculum vulgare* var. *vulgare* (Fv) and *Petroselinum crispum* (Pc) essential oils (EOs) and 11 volatile compounds on *Radix peregra* embryos (**a**), juveniles (**b**) and adults (**c**). **a** Emergence of juveniles (hatching) after 15 days of exposure to 50 mg L^{-1} of EOs/pure constituents; **b** lethality of EOs/compounds against juveniles after 24 h of exposure to 10 mg L^{-1} followed by a 24-h recovering period (48 h); **c** lethality of EOs/compounds against adults after 24 h of exposure to 50 mg L^{-1} following a 24-h recovering period (48 h). Note: The final concentrations of ethanol (negative control) were of 0.02 mL L^{-1} for assay with juveniles and 0.1 mL L^{-1} for eggs and adults. The positive control (CuSO$_4$) was tested at the same dose as EOs/compounds (10 mg L^{-1} against juveniles and 50 mg L^{-1} against eggs and adults). *I* infrutescences, *L + S* leaves plus stems, *F* fruits
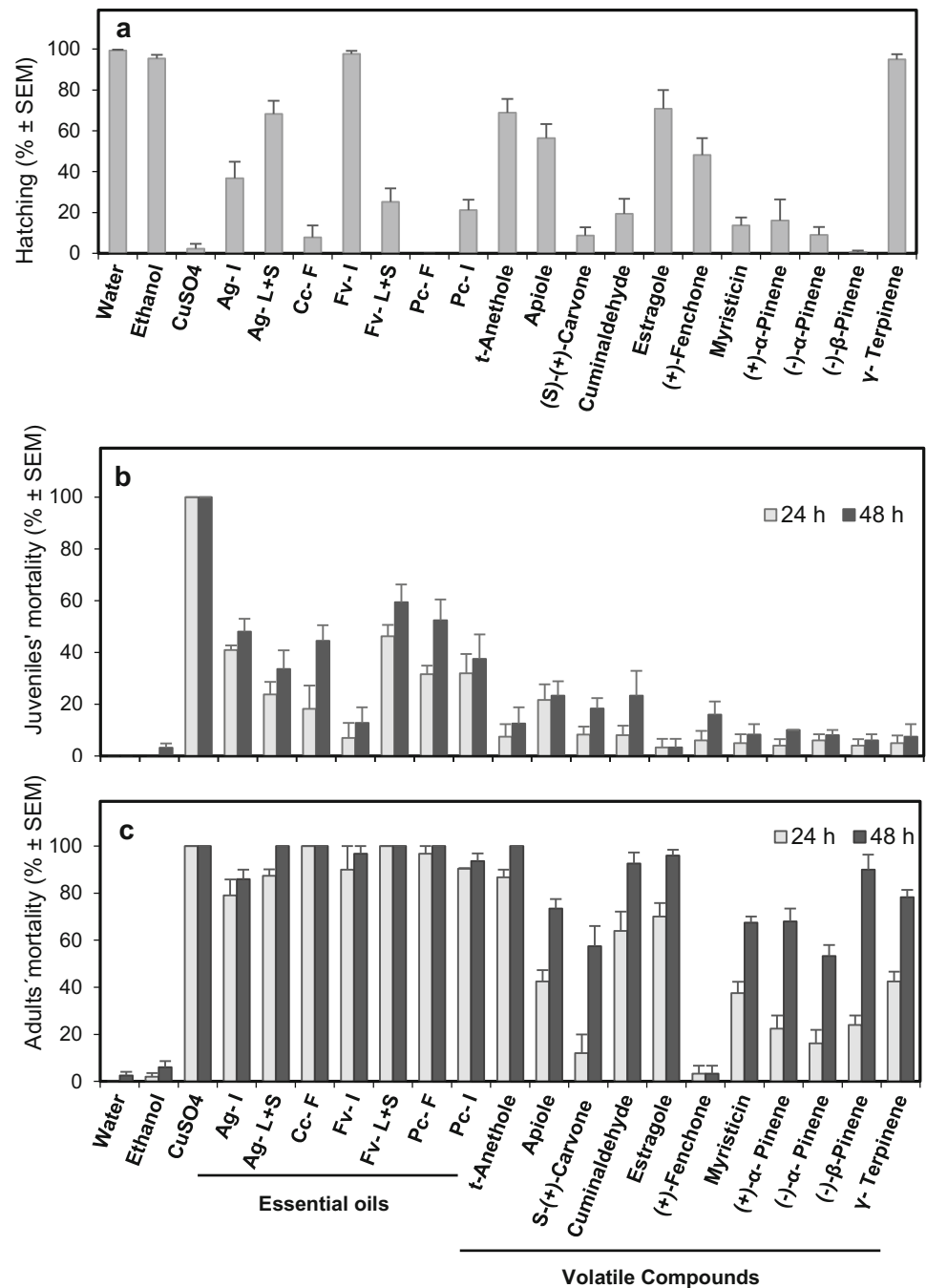
However, results obtained for the control (CuSO$_4$) and cuminaldehyde were less homogenous (higher *H*) resulting in a lower goodness of fit. Depending on the treatment, slopes of the log-probit regressions varied from 3.2 (±0.28) to 9.5 (±0.83). For the total period of the experiment (48 h), LC$_{50}$ and LC$_{90}$ values of EOs/compounds ranged from 13.7 to 46.5 and 29.2 to 83.1 mg L^{-1}, respectively.

Based on LCs values and the non-overlapping of CLs we have established, with a probability of 95%, that Pc-F was the most toxic treatment (both LCs ‹ 30 mg L^{-1}) 24 h after the interruption of the exposure, while *S*-(+)-carvone was the least toxic. By comparing the CLs of remaining treatments, we found that Pc-I, Fv-L + S, Ag-I and γ-terpinene were slightly more active than (−)-β-pinene, Ag-L + S, Fv-I, *trans*-anethole and estragole and significantly more lethal to adult snails than cuminaldehyde and Cc-F (LC$_{50}$ of 34.7 and 38.8 mg L^{-1}, respectively). Even though, when considering the LC$_{90}$ parameter, the statistical differences concerning the toxicity of these treatments were less evident.

Table 2 Estimated LC_{50} and LC_{90} of Apiaceae essential oils and some volatile compounds against adults of *Radix peregra*, after 24 h of exposure followed by a 24-h recovery period

Treatments	Conc. (mg L^{-1})	Dose-dependent lethality			
		LC_{50} (95% CL)[a]	LC_{90} (95% CL)[a]	Slope $\pm$ SEM (95% CL)[a]	H[b]
$CuSO_4$	0.1, 0.2, 0.25, 0.3, 0.5, 0.75	0.26 (0.21–0.32) a	0.65 (0.48–1.17) a	3.2 $\pm$ 0.25 (2.72–3.70) a	3.10
EOs					
Pc-F	10, 20, 30, 40	13.7 (12.1–15.1) b	29.2 (26.1–33.7) b	3.9 $\pm$ 0.36 (3.18–4.60) abc	1.80
Pc-I	10, 20, 25, 30, 40, 50	18.5 (16.9–20.0) c	40.5 (36.7–45.8) c	3.8 $\pm$ 0.30 (3.19–4.36) ab	0.52
Fv-L + S	10, 20, 30, 40, 50	21.2 (19.6–22.9) cd	42.0 (38.2–47.3) c	4.3 $\pm$ 0.33 (3.69–4.97) abc	0.57
Ag-I	20, 30, 40, 50, 60	22.3 (20.1–24.2) d	40.9 (37.3–46.3) c	4.9 $\pm$ 0.54 (3.83–5.93) bcd	1.01
Ag-L + S	20, 25, 30, 40, 50	26.4 (24.7–27.9) ef	42.6 (39.5–46.8) c	6.2 $\pm$ 0.56 (5.09–7.27) de	1.51
Fv-I	20, 25, 30, 40, 45, 50	27.5 (26.1–29.0) f	45.7 (41.2–53.3) cd	5.8 $\pm$ 0.65 (4.55–7.10) cde	2.11
Cc-F	30, 35, 40, 45	38.8 (37.6–40.3) h	53.6 (49.8–60.0) d	9.2 $\pm$ 1.08 (7.05–11.28) f	0.15
Compounds					
γ-Terpinene	10, 20, 30, 40, 50	23.1 (20.9–25.2) de	58.3 (50.6–70.4) d	3.2 $\pm$ 0.28 (2.63–3.73) a	1.69
(−)-β-Pinene	20, 30, 40, 50	26.0 (24.2–27.7) ef	44.5 (41.0–49.7) c	5.5 $\pm$ 0.52 (4.48–6.53) cd	1.79
trans-Anethole	20, 30, 40, 50	28.3 (26.4–30.1) fg	49.2 (45.0–55.5) cd	5.3 $\pm$ 0.51 (4.34–6.34) bcd	2.34
Estragole	20, 30, 35, 40, 50	28.7 (25.7–31.9) fg	44.1 (38.8–55.7) cd	7.5 $\pm$ 0.60 (5.81–8.04) def	2.11
Cuminaldehyde	30, 35, 40, 50, 60	34.7 (29.8–38.4) gh	47.4 (42.2–63.0) cd	9.5 $\pm$ 0.83 (7.89–11.15) f	3.40
S-(+)-Carvone	30, 40, 50, 60	46.5 (43.8–48.6) i	83.1 (72.8–102.3) e	4.9 $\pm$ 0.62 (3.63–6.07) abcd	1.57

[a] LC_{50}, LC_{90} values and 95% confidence limits (CL) are expressed in mg L^{-1} of essential oil/compound required to cause snails' death. LC values and slopes within the same column followed by the same letter are not significantly different based on non-overlapping of the 95% CL

[b] H, Heterogeneity factor (χ^2/df)

Furthermore, different slope values of the linear regressions have evidenced the higher sensitiveness of the snail population to small increases in the concentration of Cc-F, cuminaldehyde and estragole (Table 2). Consequently, inferior $LC_{90/50}$ ratios were obtained for Cc-F, cuminaldehyde and estragole (1.3–1.5) comparatively to remaining ones (1.5–2.5) (Online Resource 3).

Time–response

The toxicity of seven EOs and six volatiles compounds against *R. peregra* varied in function with time (Online Resource 2) and the duration of exposure (Table 3). The LT_{50} calculated for the continuous, the 16-h and the 8-h exposure varied from 15.1–34.3, 20.1–32.5 (excluding carvone) and 23.0–134.7 h, respectively, while LT_{90} varied from 21.6–49.3, 24.6–54.5 and 33.0–271 h, respectively. Moreover, the lethal parameters after 8 h of contact for Ag-I, Ag-L + S, Cc-F, *trans*-anethole, cuminaldehyde and γ-terpinene could not be estimated within a reasonable time of observation, nor within acceptable confidence limits.

With basis on the determined LT_s values (and respective 95% CLs) Ag-I, Cc-F, Fv-I, Fv-L + S, Pc-F, *trans*-anethole, cuminaldehyde and the positive control showed the acutest time-related activity on 50% of snails when these were continuously exposed ($LT_s{}_{50}$ below 22 h). With the exception of Pc-I and (S)-(+)-carvone, treatments were active against 90%

of individuals within 21.6–38.8 h of exposure (with significant differences in their time effectiveness). Regarding the 16-h exposure assay, Cc-F, Fv-I, Fv-L + S, Ag-I, *trans*-anethole, cuminaldehyde, as well as the positive control, exhibited a 90% lethal effect within a significantly lower extent of exposure time (total of 24.6–32.1 h from the beginning of the experiment) relatively to the other treatments (37.7–54.5 h). Pc-I and (S)-(+)-carvone were found to be the least active treatment (LT_{90} of 49 h) when assessed by the 16-h exposure assay. In contrast, when considering the 8-h short-term exposure, only the positive control and both bitter fennel EOs (Fv-I and Fv-L + S) were still exhibiting the most satisfactory time-related activity, causing the death of 50% of snails within 23–25.6 h (total time). In opposition, Ag-I, Ag-L + S and γ-terpinene were considerably less effective through time, with accumulated mortalities inferior to 25% after 120 h of monitoring.

Discussion

In the current work, we found that EOs from parsley and cumin fruits, and the compounds myristicin, (S)-(+)-carvone, cuminaldehyde and pinenes affected eggs hatchability the most (Fig. 1a). Our study also demonstrated that exposure of embryos to several treatments, namely Pc-F, Cc-F, myristicin and (−)-β-pinene, caused extensive death,

Table 3 Estimated LT_{50} and LT_{90} of Apiaceae essential oils (EOs) and some compounds against adults of *Radix peregra*, assessed for different exposure periods to 50 mg L^{-1}

Treatments	8-h exposure				16-h exposure				Continuous exposure			
	LT_{50} (95% CL)[a]	LT_{90} (95% CL)[a]	Slope (±SEM)	H[b]	LT_{50} (95% CL)[a]	LT_{90} (95% CL)[a]	Slope (±SEM)	H[b]	LT_{50} (95% CL)[a]	LT_{90} (95% CL)[a]	Slope (±SEM)	H[b]
$CuSO_4$[c]	25.4 (24.7–26.0) a	33.0 (31.8–34.8) a	11.3 ± 1.15 (9.1–13.6) e	1.36	20.5 (20.0–21.1) a	28.1 (26.5–30.5) a	9.4 ± 0.98 (7.5–11.3) f	1.13	20.7 (20.3–21.1) c	26.1 (25.1–27.5) c	12.6 ± 1.08 (10.5–14.7) e	0.97
Ag-I	<25%, after 120 h				21.7 (20.4–23.0) abcd	42.4 (37.8–50.1) cde	4.4 ± 0.45 (3.5–5.3) bc	1.54	21.1 (20.4–21.7) cd	31.3 (29.7–33.6) b	7.4 ± 0.52 (6.4–8.5) b	0.67
Ag-L + S	<20%, after 120 h				32.5 (27.6–38.6) f	–	1.5 ± 0.22 (1.0–1.9) a	0.39	22.8 (21.9–23.7) ef	38.6 (35.9–42.5) gh	5.6 ± 0.40 (4.8–6.4) ab	0.83
Cc-F	70%, after 140 h[d]				20.1 (19.8–20.5) a	24.6 (23.9–25.6) a	14.6 ± 1.17 (12.3–16.9) g	0.83	15.1 (14.6–15.5) a	21.6 (20.6–22.9) a	8.2 ± 0.64 (7.0–9.5) bc	0.33
Fv-I	25.7 (24.6–26.7) a	40.6 (37.5–45.9) b	6.5 ± 0.77 (5.0–8.0) d	0.86	21.4 (20.9–21.7) bc	25.6 (24.8–26.7) a	16.3 ± 1.68 (13.0–19.6) g	0.38	19.3 (18.7–19.8) b	24.1 (23.5–25.0) b	13.1 ± 1.19 (10.8–15.5) e	1.03
Fv-L + S	23.0 (12.2–26.9) a	53.0 (41.3–183.4) bc	3.5 ± 0.61 (2.4–4.7) c	2.40	21.4 (20.2–22.6) abc	26.8 (24.9–30.8) a	13.1 ± 1.0 (11.1–15.1) fg	2.05	21.0 (20.3–21.5) cd	28.6 (27.3–30.5) cde	9.5 ± 0.80 (7.9–10.9) bcde	1.55
Pc-F	60.0 (39.6–115.4) bc	–	0.6 ± 0.18 (0.2–0.9) a	0.17	25.8 (24.8–26.7) e	41.5 (38.5–45.9) cd	6.2 ± 0.54 (5.1–7.2) cd	0.17	21.6 (21.1–22.1) de	28.7 (27.7–29.9) de	10.4 ± 0.70 (9.1–11.8) cde	1.28
Pc-I	Nt				<50%, after 140 h				28.9 (27.7–30.1) h	49.2 (45.3–54.7) i	5.5 ± 0.34 (4.8–6.2) a	0.60
trans-Anethole	<35%, after 120 h				21.3 (20.7–21.9) bc	29.3 (27.8–31.7) a	9.3 ± 0.96 (7.4–11.2) ef	0.70	21.1 (20.7–21.5) cd	27.7 (26.6–29.0) cd	10.9 ± 0.71 (9.5–12.3) de	0.77
S-(+)-Carvone	Nt				98.7 (94.6–103.6) g	–	9.0 ± 1.00 (7.0–11.0) de	0.67	34.3 (33.1–35.7) i	49.3 (46.3–53.4) i	8.2 ± 0.63 (7.0–9.4) bc	1.26
Cuminaldehyde	<30%, after 120 h				20.9 (20.0–21.6) abc	32.1 (29.4–36.9) ab	6.8 ± 0.88 (5.1–8.6) cde	0.85	19.5 (18.9–20.1) b	28.4 (26.9–30.8) cde	7.9 ± 0.74 (6.4–9.3) bc	1.11
Estragole	50.4 (43.2–65.0) b	271 (159.0–775.6) c	1.7 ± 0.30 (1.2–2.3) b	0.68	24.1 (23.1–25.2) e	37.7 (34.8–42.2) bc	6.6 ± 0.63 (5.4–7.9) de	1.06	23.1 (22.4–23.9) f	32.8 (31.1–35.2) f	8.4 ± 0.60 (7.3–9.6) bcd	0.53
(−)-β-Pinene	134.7 (100.7–229.0) c	–	1.0 ± 0.16 (0.6–1.3) ab	0.07	24.4 (22.8–25.8) de	49.1 (45.1–54.7) de	4.2 ± 0.33 (3.6–5.4) bc	0.59	25.4 (24.6–26.3) g	35.6 (33.4–38.7) fgh	8.7 ± 0.67 (7.4–10.1) bcd	1.15
γ-Terpinene	<25%, after 120 h				23.2 (21.4–24.9) cde	54.5 (47.0–68.2) e	3.5 ± 0.39 (2.7–4.2) b	0.78	23.3 (22.5–24.1) f	34.9 (32.7–37.8) fg	7.32 ± 0.52 (6.2–8.4) b	0.51

Nt—not tested

[a] LT_{50}, LT_{90} values and 95% confidence limits (CL) expressed in hours required to cause death to adult snails. LT values and slopes within a column followed by the same letter are not significantly different based on non-overlapping of the 95% confidence limits (CL)

[b] H, Heterogeneity factor (χ^2: degree of freedom)

[c] For the positive control, the time–activity relationship was assessed at 0.75 mg L^{-1}

[d] LTs could not be determined due to the high heterogeneity of data

associated with a disintegrated structure, similarly to what has been described by other authors (dos Santos and Sant' Ana 2001). Accordingly, we infer that the 50 mg mL^{-1} of these EOs/compounds displays an effective biocidal activity against snails' embryos by disrupting embryogenesis. Although there are several aspects that may possibly have great influence on these results, it is most likely that embryos' susceptibility to extracts/natural compounds is intrinsically related to the physical and chemical properties of each individual constituents, their ability to disperse into the gelatinous matrix of the egg mass and the nature of interactions (between compounds and the tissues/molecular targets). It is recognized that the lipophilic character of fragrance compounds and their affinity towards lipid-rich tissues is mainly responsible for the broad spectrum of biological activities (Lahlou 2004).

The EOs from dill, parsley, cumin and bitter fennel (L + S), as well as the compound cuminaldehyde and apiole, were the most active against both young and adult snails (Fig. 1b, c, respectively). On the other hand, we observed that lethality of treatments over juveniles and adults could differentially increase from the onset to the end of the recovery period (24 and 48 h) (Fig. 1b, c), as mortality rates of newly hatched snails and adults increased 1.2–3.2 times and 1.2–4.8 times, respectively (Online Resource 1). Several reports have shown that snails can be differentially susceptible to chemicals depending on their developmental stages. For instance, *Euphorbia splendens* and *Phytolacca dodecandra* are toxic to snails but have a low ovicidal activity at levels that are lethal to adults (McCullough et al. 1980; Schall et al. 1998). On the other hand, young snails of *R. peregra* and *Biomphalaria alexandrina* are more susceptible than elder ones to some plant EOs and methanol extracts, respectively (Teixeira et al. 2011; Bakry 2009). However, activity against both adult and egg stages is of upmost importance for an efficient control of host snail populations.

A time-dependent study was previously performed against *R. peregra* with EOs from plant species of the Macaronesia flora found in Azores (Teixeira et al. 2011). Therefore, we consider that a suitable assessment of molluscicidal activity of a given extract or compound may involve the respective dose-related evaluation associated with a time-dependent effect. Under this perspective, we conclude that Pc-F was the most effective product regarding the dose activity criteria, though its lethality was more lagged in time (Table 3). On the contrary, when compared to Pc-F, Cc-F induced its highest lethality level within short times despite its much lower dose-dependent lethality, as LC$_{50}$ and LC$_{90}$ values for Cc-F were 2.8 and 1.8 times greater, respectively, than Pc-F. Apparently, Cc-F impairs vital mechanisms more rapidly than Pc-F, resulting in overall failure of the organism. According to Price and Berry (2008), millimolar concentrations of EOs compounds have a blocking effect on action potentials, besides impairing the response to some neurotransmitters (octopamine, dopamine and acetylcholine). For instance, the compound myristicin, extracted from *M. fragrans* and herein found in parsley fruits (31.5%), was highly toxic to *L. acuminata* (LC$_{50}$ ≤10 mg L^{-1}) and established as a potent inhibitor of acetylcholinesterase (IC = 0.03 mM) and of acid phosphatase (0.07 mM) in (Jaiswal and Singh 2009). Even so, as it is true for most insecticides, the mode of action of a molluscicide should be a multi-component process affecting more than one system, which culminates in the death of the organism (McCullough et al. 1980; WHO 1983).

In general, EOs are recognized as non-persistent plant products owing to the low molecular weight (high volatility) and biodegradability of their constituents (Regnault-Roger et al. 2012). Changes of chemical profile over time are one of the many aspects that might have a severe impact on their biological effects and the overall effectiveness (Isman and Miresmailli 2010). Like any natural or synthetic compound, reliable results under field conditions might be compromised by the action of the biota. For example, extracts of *P. dodecandra* (Phytolaccaceae) and its active compound (oleanolic acid glucoside) are rapidly biodegraded with a consequent loss of the molluscicidal potency after a few days (McCullough et al. 1980). Likewise, depletion of some molluscicides (like niclosamide and copper-based compounds) takes place soon after their application into water (Muir and Yarechewski 1982; Francis-Floyd et al. 1997). Even though this could represent an advantage regarding environmental pollution, such rapid degradation may be a constraint for the application of molluscicides from natural origins. Eventually the use of controlled delivery device would be a good strategy to improve their solubility, ensure their stability and the slow release of these products. In addition, products application should also be examined regarding the potential development of heritable resistances under field conditions, especially if there is evidence of a large percentage of survival. Therefore, in this study we questioned whether there were possible differences in the recovering dynamics of *R. peregra* snails and opted to explore eventual loss of effectiveness when the exposure was shorter. In most cases (except for carvone and Pc-I) we confirmed that a 16-h exposure at a concentration of 50 mg L^{-1} might be sufficient to reduce 90% of *R. peregra* adult population (Table 3). Nevertheless, we verified that survival percentages following the 8-h exposure test were undesirably high for the majority of EOs/compounds (respective estimated LTs were moderately to considerably superior and statistically distinct to their counterparts determined for the other exposure times assays). Under these conditions (after 8 h of contact), only the bitter fennel EOs characterized herein (rich in estragole, fenchone and α-pinene and α- and β-phellandrenes) maintained their

effectiveness at the tested concentration (Table 3). Indeed, a 8-h period of exposure to dill EOs, *trans*-anethole, cuminaldehyde or γ-terpinene (at 50 mg L^{-1}) was not enough to cause acute toxicity and high mortality percentage in adults. In what concerns dill EOs, *trans*-anethole, cuminaldehyde and γ-terpinene our findings suggest that a 8-h period of contact was not enough to cause an irreversible state of toxicity and unrecoverable injuries to snails' organism. We suppose that under the tested circumstances most individuals exposed to these natural plant products for 8 h were able to recover when transferred to clear water. These findings are consistent with those of Price and Berry (2008) who reported the reversible knockdown effects of the volatile compounds citral, geraniol and eugenol on *Planorbis corneus* after a brief 5-h exposure to acute lethal doses. The uptake and progressive accumulation of the active compound(s) into the snail body and/or their metabolization into more toxic forms by the organism enzymatic system have been suggested as possible events responsible for time-related toxicity (Kumar and Singh 2006; Jaiswal and Singh 2009).

In this work, we noticed that higher dosages elicited acute symptoms (increased mucous secretion and unresponsiveness to stimuli) in the first hours of exposure to EOs and compounds. Those symptoms were followed by swelling of the cephalopedal mass with haemolymph leakage, protrusion of the snail body, discoloration of the tissues and disintegration of the foot epithelium (Online resource 4). Such behavioural responses are in accordance with those described by several authors for pulmonate snails exposed to different plant extracts and constituents (McCullough et al. 1980; Lahlou and Berrada 2001; Price and Berry 2008). On the contrary, we observed that snails exposed to copper salt manifested strong avoiding behaviour by attempting to evade the solution and, in a second phase, an absence of responsiveness. It is worth mentioning that the activity of this copper-based molluscicide against *R. peregra* (LCs of CuSO$_4$ ‹1 mg L^{-1}) recorded herein was significantly higher than that of EOs and volatiles compounds (LC$_{90}$ was 45–128 times superior). The LC$_{50}$ of copper sulphate on *R. peregra* was very close to that determined against *B. glabrata* (48 h LC$_{50}$ = 0.48 mg L^{-1}) (de Oliveira-Filho et al. 2004).

The biocidal effects observed for EOs in each assay were somewhat consistent with the activity determined for the compounds tested. Cc-F and cuminaldehyde activities were coherent in terms of LCs, with parallel dose–mortality rates (same slopes) (Table 2), and ovicidal activity (Fig. 1a). Thus, cuminaldehyde reflected EO activity in both aspects, while γ-terpinene did not. This finding strongly indicates that most of the Cc-F activity is likely attributable to this major compound (cuminaldehyde) found at 39%, but also to β-pinene (12%), rather than to γ-terpinene, which constitute 16% of the cumin EO

(Table 1). Nonetheless, we cannot rule out that other compounds (i.e. *p*-cymene, *p*-mentha-1,4-dien-7-al, etc.) comprised in the remaining part of the EO (1/3) could play a role in the dose and time effectiveness observed for the cumin fruits EO, by acting synergistically with cuminaldehyde. Interestingly, Pc-F and Pc-I showed comparable toxicity in terms of LCs and dose-dependent increases in snails' mortality (Table 2), despite the considerable differences in their composition (Table 1). The results obtained in the single-dose assays indicate that the ovicidal activity of Pc-F was influenced by the pinenes and myristicin activities, while apiole seemed to have had a higher impact in the toxicity of parsley EOs against adults than against embryos (Fig. 1a, c). Contrasting results were also obtained for bitter fennel EOs, since Fv-L + S was significantly more active than Fv-I against eggs and juveniles. On the other hand, estragole was effective against adults but almost inactive towards eggs and juveniles (Fig. 1). This could explain, to a certain extent, why both EOs only showed equivalent effectiveness against the adult form regardless of their distinct quantitative profiles. This relation is evidenced by the coherent values of LCs and regression slopes exhibited by Fv-I EO and its main constituent estragole (65%), as shown in Table 2. On the contrary, we have found that the dose- and time-dependent toxicity of Ag-I against adults was not very consistent with the toxicity of (*S*)-(+)-carvone, which represents the major part of this EO (2/3), whereas both were coherent in terms of ovicidal activity. These results also evidence that *R. peregra* susceptibility to Ag-I and (*S*)-(+)-carvone is variable depending on the developmental stage and that toxicity towards adults is more likely to be attributed to other non-tested compounds, which were found in considerable amounts in both dill EOs, or a combination of these (α- and β-phellandrene and limonene accounted for 70 and 28% in leaves plus stems and infrutescences EOs, respectively). Little is known about the molluscicidal activity of phellandrenes, but limonene has been previously reported as a good molluscicide against *L. acuminata* (Kumar and Singh 2006; Agrahari and Singh 2013).

Conclusion

The present findings evidenced the strong potential of the tested EOs/compounds against the trematodes intermediate host, *R. peregra*. Interestingly, our results show that *R. peregra* susceptibility to EOs and compounds is variable and visibly reliant on its developmental stage. The estimated LC$_{50}$ of the tested EOs (13.7–38.8 mg L^{-1}) was below the threshold value of 40 mg L^{-1} that distinguishes the plant crude extracts with the highest molluscicidal potential and accordingly eligible to be employed against

mollusc populations (WHO 1993, 1995; Singh et al. 1996). One key question in our work was: how long should the exposure to some of the most dose-active treatments last to trigger irreversible acute symptoms? Our results indicate that effectiveness can be achieved within a short time (at least 16 h of exposure for most treatments), which is clearly an appealing aspect of this type of alternative product, considering its high biodegradability and volatile nature. Moreover, the ovicidal effect recorded for EOs at levels that are lethal to adult snails is of great advantage. Thus, we suggest that the application of the studied EOs as natural molluscicidal agents could be a profitable natural alternative considering their significant bioactivity in a time-effective mode, the EO productivity of these plant species and the availability of these products in the market. For some EOs, the bioactivity recorded was related to the activity of the compounds included in the study. Therefore, we could consider the use of these constituents in artificial mixture in combination with other compounds to potentiate their activity. As a perspective for future work, and being aware of the influenced of several factors on the efficacy of EOs, we shall consider to perform some assays concerning the physical chemical stability of these products. Further studies, making use of controlled release systems, which are already been applied in some commercialized pesticides, are also under consideration.

Author Contributions MF-F (coordinator), AC and JR provided the reagents and tools. RMS and JR designed and performed the experiments and data analysis. The manuscript was drafted by RMS. All authors reviewed and approved the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Human and animal rights Not applicable.

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