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First report on *Meloidogyne chitwoodi* hatching inhibition activity of essential oils and essential oils fractions

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Abstract The Columbia root-knot nematode (CRKN), *Meloidogyne chitwoodi*, is an EPPO A2 type quarantine pest since 1998. This nematode causes severe damage in economically important crops such as potato and tomato, making agricultural products unacceptable for the fresh market and food processing. Commonly used nematicidal synthetic chemicals are often environmentally unsafe. Essential oils (EOs) may constitute safer alternatives against RKN. EOs, isolated from 56 plant samples, were tested against CRKN hatching, in direct contact bioassays. Some of the most successful EOs were fractionated and the hydrocarbon molecules (HM) and oxygen-containing molecules (OCM) fractions tested separately. 24 EOs displayed very strong hatching inhibitions (≥90 %) at 2 μL mL^{-1} and were further tested at lower concentrations. *Dysphania ambrosioides*, *Filipendula ulmaria*, *Ruta graveolens*, *Satureja montana* and *Thymbra capitata* EOs revealed the lowest EC_{50} values (<0.15 μL mL^{-1}). The main compounds of these EOs, namely 2-undecanone, ascaridol, carvacrol, isoascaridol, methyl salicylate, *p*-cymene and/or γ-terpinene, were putatively considered responsible for CRKN hatching inhibition. *S. montana* and *T. capitata* OCM fractions showed hatching inhibitions higher than HM fractions. The comparison of EO and corresponding fractions EC_{50} values suggests interactions between OCM and HM fractions against CRKN hatching. These species EOs showed to be potential environmentally friendly CRKN hatching inhibitors; nonetheless, bioactivity should be considered globally, since its HM and OCM fractions may contribute, diversely, to the full anti-hatching activity.

Keywords Columbia root-knot nematode · *Dysphania ambrosioides* · *Filipendula ulmaria* · *Ruta graveolens* · *Satureja montana* · *Thymbra capitata*

Key message

- This study identified five very strong hatching inhibitor EOs isolated from *Dysphania ambrosioides*, *Filipendula ulmaria*, *Ruta graveolens*, *Satureja montana* and *Thymbra capitata*.
- Fractionation and testing suggested that fractions containing hydrocarbon compounds (HM) and oxygenated compounds (OCM) may each contribute to the full anti-hatching activity.

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Introduction

Root-knot disease is caused by plant parasitic nematodes of the genus *Meloidogyne* and is characterized by the presence of galls or knots in the roots below ground and stunted growth, yellowing of the leaves, lack of vigour, a tendency to wilt under moisture stress and collapse of individual plants above ground that are similar to other root diseases. These symptoms are due to infection which mobilizes the plant's photosynthates from shoots to roots and affects water and nutrient absorption and translocation in the root system to support nematode development and reproduction (EPPO 2012). The Columbia root-knot nematode (CRKN), *Meloidogyne chitwoodi* Golden et al. (1980), a sedentary and obligate plant endoparasite, is responsible for large economic losses in several horticultural and field crops and has been classified, in 1998, as an A2 type quarantine pest by the European Plant Protection Organization (EPPO 2012). Since its first description in the Pacific Northwest, USA, several reports have been made in South Africa, Belgium, Germany, the Netherlands, Portugal, Mexico and Argentina (Golden et al. 1980; Conceição et al. 2009; OEPP, EPPO 2009; Wesemael et al. 2011). In potato, tuber infection is characterized by the presence of galls, small raised swellings on the surface above the developing nematodes, and necrosis and browning of the internal tissue below the gall. When 5 % or more of the tubers are tarnished, the crop is usually unmarketable (EPPO 2012). Being the world's fourth-largest food crop (FAO 2009), potato production employs large quantities of pesticides, mainly synthetic chemicals applied by soil fumigation (e.g. 1,3-dichloropropene, methyl bromide, dazomet or nervous system toxins such as oxamyl and fenamiphos) (Mitkowski and Abawi 2003). Although highly efficient in controlling this soil pest (Pinkerton et al. 1986), fumigation has a negative environmental impact, making the continued availability and use of soil fumigants uncertain. In face of the recent EU environmental restrictions, it is necessary to develop environmentally safer control techniques based upon natural products. Essential oils (EOs) may prove to be sound alternatives to synthetic nematicides. They are complex mixtures of volatiles, mainly products from the plant secondary metabolism, comprised terpenes, mostly mono-, sesqui- and diterpenes, and phenolic compounds, such as phenylpropanoids, although other groups of compounds can also occur in relevant amounts. Generally biodegradable, EOs have low toxicity to mammals and do not accumulate in the environment (Figueiredo et al. 2008). Moreover, the biological activities of EOs can often exceed the sum of their single constituent's activities, due to synergy (Ntalli et al. 2011a; Kumrungsee et al. 2014). As complex mixtures, EOs may display several biological activities which make them desirable biopesticides (Batish et al. 2008) able of controlling not only the targeted pest but also opportunistic species and resistant strains.

No studies on the effect of EOs against *M. chitwoodi* have been conducted, but a strong anti-nematode activity, against other *Meloidogyne* species, was found in several EOs, such as those of *Allium sativum*, *Carum capticum, C. carvi*, *Chrysanthemum coronarium*, *Eucalyptus globulus*, *Foeniculum vulgare*, *Mentha rotundifolia*, *M. spicata*, *Origanum majorana*, *Pimpinella anisum* and *Syzygium aromaticum,* and among others (Oka et al. 2000; Pérez et al. 2003; Ibrahim et al. 2006; Meyer et al. 2008; Douda et al. 2010; Gupta et al. 2011; Ntalli et al. 2011a; Andrés et al. 2012).

In an attempt to clarify the nematotoxic potential of EOs against different types of nematodes and their value to sustainable pest control, a previous study addressed the nematotoxicity of several EOs against a different nematode type, the pinewood nematode, *Bursaphelenchus xylophilus* (Faria et al. 2013). To our best knowledge, no previous study has addressed the activity of EOs against CRKN hatching. In view of the increasing potato demand and the need for environmentally safer anti-nematode compounds, the present study aimed at performing a comparative screening of EOs to (a) determine, through direct contact bioassays, those that show high hatching inhibition and (b) assess the relative importance of EOs hydrocarbon- and oxygen-containing molecules (OCM) fractions in *M. chitwoodi* hatching inhibition.

Materials and methods

Nematodes

The CRKN eggs, used in the bioassays, were obtained from previously established *Solanum tuberosum* hairy roots with *M. chitwoodi* co-cultures (Faria et al. 2014). Subculture was performed monthly by refreshment of the culture medium. Approximately 5 g (fresh weight) of co-culture was transferred to 200 mL SH liquid medium (Schenk and Hildebrandt 1972), supplemented with 30 g L^{-1} sucrose, pH 5.6, maintained in darkness at 24 ± 1 °C on orbital shakers (80 rpm). After 3 months of subculture, galled hairy roots were excised and the CRKN eggs extracted by a 5-min immersion in a 0.52 % (v/v) sodium hypochlorite (NaOCl) solution, with vigorous agitation (Hussey and Barker 1973). Eggs were collected in a 20-µm mesh sieve, rinsed thoroughly with ultrapure water, to remove NaOCl traces, quantified and used directly in the bioassays. Nematode and egg counting were performed using an inverted microscope [Diaphot, Nikon, Japan (×40)].

Plant material, essential oils and essential oil fractions

Collective and/or individual samples, from cultivated and wild-growing medicinal and aromatic plants, were collected from mainland Portugal and at the Azores archipelago (Portugal) (Table 1). Dried aerial parts from commercially available products sold in local herbal shops were also analysed. A total of 56 samples from sixteen families were tested. A voucher specimen of each plant species, collected from wild state condition, was deposited in the Herbarium of the Botanical Garden of Lisbon University, Lisbon, Portugal. For commercial plant material, a reference sample from each plant is retained at the CBV laboratory and is available upon request.

Essential oils were isolated by hydrodistillation for 3 h using a Clevenger type apparatus according to the European Pharmacopoeia (Council of Europe 2010). Hydrodistillation was run at a distillation rate of 3 mL min^{-1} and EOs stored in the dark at −20 °C, until analysis. Fractions containing hydrocarbons molecules (HM) or OCM were separated from each EO sample on a silica gel column by elution with distilled *n*-pentane and diethyl ether, respectively, as previously detailed (Faria et al. 2013).

Analysis of volatiles

Volatiles were analysed by gas chromatography (GC), for component quantification, and GC coupled to mass spectrometry (GC–MS) for component identification. Gas chromatographic analyses were performed using a Perkin Elmer Autosystem XL gas chromatograph (Perkin Elmer, Shelton, CT, USA) equipped with two flame ionization detectors, a data handling system and a vaporising injector port into which two columns of different polarities were installed: a DB-1 fused-silica column (30 m × 0.25 mm i.d., film thickness 0.25 μm; J & W Scientific Inc., Rancho Cordova, CA, USA) and a DB-17HT fused-silica column (30 m × 0.25 mm i.d., film thickness 0.15 μm; J & W Scientific Inc., Rancho Cordova, CA, USA). Oven temperature was programmed to increase from 45 to 175 °C, at 3 °C min^{-1} increments, then up to 300 °C at 15 °C min^{-1} increments, and finally held isothermal for 10 min. Gas chromatographic settings were as follows: injector and detectors temperatures, 280 and 300 °C, respectively; carrier gas, hydrogen, adjusted to a linear velocity of 30 cm s^{-1}. The samples were injected using a split sampling technique, ratio 1:50. The volume of injection was 0.1 μL of a pentane-oil solution (1:1). The percentage composition of the oils was computed by the normalization method from the GC peak areas, calculated as a mean value of two injections from each volatile oil, without response factors.

The GC–MS unit consisted of a Perkin Elmer Autosystem XL gas chromatograph, equipped with DB–1 fused-silica column (30 m ×0.25 mm i.d., film thickness 0.25 μm; J & W Scientific, Inc., Rancho Cordova, CA, USA) interfaced with Perkin-Elmer Turbomass mass spectrometer (software version 4.1, Perkin Elmer). GC–MS settings were as follows: injector and oven temperatures were as above; transfer line temperature, 280 °C; ion source temperature, 220 °C; carrier gas, helium, adjusted to a linear velocity of 30 cm s^{-1}; split ratio, 1:40; ionization energy, 70 eV; scan range, 40–300 u; scan time, 1 s. The identity of the components was assigned by comparison of their retention indices relative to C_8–C_{25} *n*-alkane indices, and GC–MS spectra from a laboratory made library based upon the analyses of reference oils, laboratory-synthesized components and commercial available standards.

The percentage composition of the isolated EOs was used to determine the relationship among the samples by cluster analysis using Numerical taxonomy multivariate analysis system (NTSYS-pc software, version 2.2, Exeter Software, Setauket, New York) (Rohlf 2000). For cluster analysis, correlation coefficient was selected as a measure of similarity among all accessions and the unweighted pair group method with arithmetical averages (UPGMA) was used for cluster definition. The degree of correlation was evaluated, according to Pestana and Gageiro (2000), as very high (0.9–1), high (0.7–0.89), moderate (0.4–0.69), low (0.2–0.39) and very low (<0.2).

Bioassays

All bioassays were performed in flat bottom 96-well microtiter plates (Carl Roth GmbH + Co. KG, Karlsruhe, Germany). EOs, hydrocarbon molecules (HM) and OCM fractions were assayed in suspensions of newly extracted mixed-developmental stage *M. chitwoodi* eggs, using a methodology adapted from Faria et al. (2013). Egg suspensions were chosen for experimentation, instead of egg masses, to ensure that EO concentration was homogenous for all eggs. Aliquots with 99 μL of a suspension of eggs (80–100) were introduced to each well, and 1 μL of EOs or fractions stock solutions prepared in methanol (Panreac Química S.A.U., Barcelona, Spain), at 200 μL mL^{-1}, was added, being the highest concentration tested 2 μL mL^{-1}. Stock solutions for 1, 0.5, 0.25 and 0.125 μL mL^{-1} were obtained by serial dilutions with a dilution factor of two. The EOs and fractions which showed hatching inhibitions <90 % were not further assayed at lower concentrations. Controls were performed with methanol, 1 % (v/v, methanol/egg suspension) and ultrapure water was used to check the hatching inhibition induced by methanol. The plates were covered to diminish EO volatilization, wrapped with aluminium foil to establish total darkness and

Table 1 Plant family and species, sampling year, plant part used for hydrodistillation, plant source, essential oil (EO) yield and main components (≥10 %)

Family/Species	Code	Sampling date	Plant part[a]	Collection place/source	Yield (%, v/w)	Main components (%)
Amaranthaceae						
Dysphania ambrosioides (L.) Mosyakin and Clemants	Da	2013	FF	Évora	0.56	Isoascaridol 51, ascaridol 16
Apiaceae (Umbelliferae)						
Foeniculum vulgare Mill. 1[b]	Fv1	2008	FF	Graciosa, Azores	0.33	*trans*-Anethole 73, α-pinene 13
Foeniculum vulgare Mill. 2	Fv2	2013	Seeds	Herbal shop	1.16	Methyl chavicol 79, limonene 12
Petroselinum crispum (Mill.) Nym.[b]	Pc	2009	FV	Lisbon	0.09	1,3,8-*p*-Menthatriene 50, β-myrcene 13, apiole 11
Asteraceae (Compositae)						
Achillea millefolium L.[b]	Am	2010	DF	Herbal shop	0.85	β-Thujone 33, *trans*–chrisantenyl acetate[e] 19
Solidago virgaurea L.	Sv	2013	FF	Setúbal	0.72	β-Pinene 22, α-pinene 21, germacrene D 15, limonene 12
Cupressaceae						
Cryptomeria japonica (Thunb. ex L.f.) D. Don[b]	Cj	2008	Ffruit	Flores, Azores	0.41	Terpinen-4-ol 24, α-pinene 23, sabinene 17
Fabaceae (Leguminosae)						
Genista tridentata L.[b]	Gt	2010	DV	Herbal shop	<0.05	*cis*-Theaspirane 27, *trans*-theaspirane 22
Geraniaceae						
Pelargonium graveolens L'Hér.[b]	Pg	2009	FV	Lisbon	0.19	Citronellol 34, guaia-6,9-diene 15, citronellyl formate[e] 14
Lamiaceae (Labiatae)						
Calamintha nepeta (L.) Savi[b]	Cn	2009	FF	Castelo Branco	1.43	Isomenthone 52, isomenthol 19, 1,8-cineole 11
Lavandula luisieri (Rozeira) Rivas Mart.[c]	Ll	2013	DF	Herbal shop	0.44	5-Methylene-2,3,4,4-tetramethylcyclopent-2-enone 18, 1,8-cineole 16
Mentha arvensis L.[b]	Ma	2009	FV	Lisbon	0.06	Piperitenone oxide 56
Mentha cervina L.[b]	Mc	2009	DV	Castelo Branco	0.80	Pulegone 80
Mentha x piperita L. 1[b]	Mp1	2009	FV	Lisbon	0.11	Menthol 31, menthone 19
Mentha x piperita L. 2[b]	Mp2	2009	FV	Lisbon	0.73	Menthone 56, pulegone 13
Mentha pulegium L.[b]	Mpu	2008	DV	Lisbon	0.35	Pulegone 49, piperitenone 10
Mentha spicata L.[b]	Ms	2009	FV	Lisbon	0.07	Carvone 54
Nepeta cataria L.[b]	Nc	2009	FF	Herbal shop	0.18	4aα, 7α, 7aα-Nepetalactone 89
Origanum majorana L.	Om	2013	DV	Herbal shop	0.90	Terpinen-4-ol 18, carvacrol 17, γ-terpinene 13, methyl carvacrol 13
Origanum vulgare L.[b]	Ov	2010	DL	Herbal shop	1.00	α-Terpineol 16, thymol 15, γ-terpinene 15, carvacrol 10
Origanum vulgare subsp. *virens* (Hoffmanns. & Link) Bonnier and Layens[b]	Ovi	2010	DV	Herbal shop	0.83	α-Terpineol 40, linalool 16, thymol 12
Rosmarinus officinalis L.[b]	Ro	2009	DL	Herbal shop	1.95	β-Myrcene 29, α-pinene 15
Salvia officinalis L.	So	2010	FV	Herbal shop	1.20	1,8-Cineole 13, borneol 12, α-humulene 12
Satureja montana L. 1[b]	Sm1	2010	DF	Herbal shop	1.31	Carvacrol 64, γ-terpinene 18

Table 1 continued

Family/Species	Code	Sampling date	Plant part[a]	Collection place/source	Yield (%, v/w)	Main components (%)
Satureja montana L. 2	Sm2	2013	DV	Herbal shop	1.48	Carvacrol 77
Thymbra capitata (L.) Cav.[b]	Tc	2010	FF	Algarve	1.40	Carvacrol 68, γ-terpinene 11
Thymus caespititius Brot. 1[b]	Thc1	2008	FF	Gerês	0.35	α-Terpineol 36, *p*-cymene 13, γ-terpinene 13
Thymus caespititius Brot. 2[b]	Thc2	2008	FF	Graciosa, Azores	0.38	α-Terpineol 62
Thymus caespititius Brot. 3[b]	Thc3	2009	FF	Terceira, Azores	0.33	Thymol 42, thymol acetate15, *p*–cymene 14
Thymus caespititius Brot. 4[b]	Thc4	2004/09	FF	Azores	[d]	Carvacrol 54, carvacrol acetate 10
Thymus caespititius Brot. 5[b]	Thc5	2010	FF	Coimbra	0.48	Carvacrol 59, *p*-cymene 11
Thymus pulegioides L.	Thp	2013	DV	Herbal shop	0.49	Thymol 32, *p*-cymene 22
Thymus villosus subsp. *lusitanicus* (Boiss.) Coutinho [b]	Thvl	2008	FF	Leiria	1.25	Linalool 69
Thymus vulgaris L.	Thv	2013	DV	Herbal shop	1.20	Thymol 45, *p*-cymene 21, γ-terpinene 16
Thymus zygis subsp. *silvestris* (Hoffmanns. and Link) Coutinho[b]	Thzs	2008	FF	Santarém	0.94	α-Terpineol 60
Thymus zygis Loefl. ex L. subsp. *zygis*	Thzz	2013	FF	Bragança	0.71	Carvacrol 45, *p*-cymene 22, γ-terpinene 17
Lauraceae						
Cinnamomum camphora (L.) Sieb[b]	Cc	2009	FF	Coimbra	0.47	Camphor 49, α-pinene 10
Laurus azorica (Seub.) J. Franco[b]	La	2008	FV	Flores, Azores	0.25	α-Pinene 35, β-pinene 16, *trans*-α-bisabolene 15
Laurus nobilis L.[b]	Ln	2009	DL	Herbal shop	0.95	1,8-Cineole 35, α-terpenyl acetate 13
Myristicaceae						
Myristica fragrans Houtt.	Mf	2013	Macis	Herbal shop	2.60	Safrole 41, terpinen-4-ol 11, sabinene 10
Myrtaceae						
Eucalyptus citriodora Hook.[b]	Ect	2009	FV	Santarém	0.86	Citronellal 36, isopulegol 13, citronellol 12, 1,8-cineole 11
Eucalyptus dives Schauer[b]	Ed	2009	FV	Santarém	3.30	Piperitone 40, α-phellandrene 19, *p*-cymene 19
Eucalyptus globulus Labill.	Eg	2009	FV	Lisbon	3.02	1,8-Cineole 65, α-pinene 20
Eucalyptus smithii R.T. Baker[b]	Esm	2009	FV	Santarém	2.80	1,8-Cineole 83
Eucalyptus urophylla S. T. Blake[b]	Eu	2009	FV	Santarém	0.86	α-Phellandrene 45, 1,8-cineole 23
Syzygium aromaticum (L.) Merrill and Perry [b]	Sa	2010	Dfb	Herbal shop	9.00	Eugenol 92
Pinaceae						
Pinus halepensis Mill.	Ph	2010	FV	Algarve	0.21	α-Pinene 32, β-myrcene 29
Poaceae (Gramineae)						
Cymbopogon citratus (DC) Stapf.[b]	Cci	2010	DL	Herbal shop	3.04	Geranial 34, neral 22, β–myrcene 20, geraniol 18
Rosaceae						
Filipendula ulmaria (L.) Maxim.	Fu	2013	DV	Herbal shop	0.10	Methyl salicylate 85
Rutaceae						
Citrus limon (L.) Burm. f. Var. Meyer[b]	Cl	2009	Fex	Algarve	0.25	Limonene 45, 1,8-cineole 15, β-pinene 14

Table 1 continued

Family/Species	Code	Sampling date	Plant part[a]	Collection place/source	Yield (%, v/w)	Main components (%)
Citrus sinensis (L.) Osbeck 1[b]	Cs1	2009	Ffl	Lisbon	0.14	Sabinene 47, limonene 10
Citrus sinensis (L.) Osbeck 2[b]	Cs2	2009	FV	Lisbon	0.26	Sabinene 64
Citrus sinensis (L.) Osbeck var Valencia Late 3[b]	Cs3	2009	Fex	Algarve	0.45	Limonene 78, *β*-phellandrene 13
Ruta graveolens L.[b]	Rg	2010	DV	Herbal shop	0.51	2-Undecanone 91
Verbenaceae						
Aloysia citrodora Gómez Ortega and Palau[b]	Ac	2009	DV	Herbal shop	0.19	Geranial 12, limonene 11, neral 10
Zingiberaceae						
Zingiber officinale Roscoe[b]	Zo	2008	Frhiz	Herbal shop	0.16	Geranial 29, *β*-phellandrene 17, citronellol 14, camphene 14

[a] *DF* dry flowering phase aerial parts, *Dfb* dry flower buds, *DL* dry leaves, *DV* dry vegetative phase aerial parts, *Fex* fresh exocarp, *FF* fresh flowering phase aerial parts, *Ffl* fresh flowers, *Ffruit* fresh fruit, *FL* fresh leaves, *Frhiz* fresh rhizome, *FV* fresh vegetative phase aerial parts

[b] Detailed composition of EOs reported in Faria et al. (2013)

[c] Commercialized as *Lavandula stoechas* L.

[d] EO resulted from the combination of several EOs from the same chemotype collected in Azores from 2004 to 2009

[e] Identification based on mass spectra only

maintained at 27 ± 1 °C. Hatched second-stage juvenile nematodes (J2) were counted every 24 h during three days (72 h). A minimum of 10 replicates was performed for each sample, in, at least, two separate assays.

Determination of hatching inhibition percentages and EC_{50} values

Hatching rates (J2 day^{-1}) were obtained by fitting a linear regression to the cumulative time-course hatching data. Slope (m) values, corresponding to hatching rates, were used to determine the corrected hatching inhibition (CHI) through an adaptation of the Abbott formula (Abbott 1925), corrected hatching inhibition (CHI) % = $[1–(m_{treatment}/m_{control})] \times 100$.

Classification of the EOs and fractions hatching inhibition activity was adapted from Dias et al. (2012) in very strong (≥90 %) strong (60–89 %), moderate (37–59 %), weak (11–36 %) and low or inactive (<10 %).

Effective doses which resulted in 50 % hatching inhibition (EC_{50}) were determined using the mean corrected hatching inhibition percentage values. These data were subjected to non-linear regression analysis using a dose–response log-logistic equation (Seefeldt et al. 1995):

$$y = C + (D-C)/1 + \exp\{b[\log(x)-\log(EC_{50})]\},$$

which relates the average response y to dose x, and where C and D are, respectively, the lower- and the upper limit of the sigmoidal dose–response curve and b is the slope. This analysis was performed using GraphPad Prism® version 5.00 for Windows, San Diego California USA (www.graphpad.com), setting C to 0 % and D to 100 % with variable slope (b).

Results

Essential oils CRKN hatching inhibition

CRKN hatching inhibition percentages were evaluated through direct contact bioassays. Control assays were performed with ultrapure water and pure methanol, used as EO solvent. The average hatching rate of the controls ultrapure water and methanol was 5.0 ± 0.4 J2 day^{-1} and a 4.5 ± 0.3 J2 day^{-1}, respectively. Hatching inhibition due to methanol, in the concentration 1 % (v/v), was considered negligible.

Herewith, 42 of the EOs previously tested against *B. xylophilus* motility (Faria et al. 2013) plus 14 new EOs were comparatively assessed against *M. chitwoodi* hatching. All 56 EOs were fully chemically characterized, although Table 1 reports only their main components (≥10 %). The full chemical composition of 42 samples was previously reported in Faria et al. (2013) supplementary Table, and the 14 new EOs full compositions are detailed in the current

Supplementary Table, ST. Cluster analysis was performed on the EOs and EOs fractions full composition to identify groups of similar EO volatile patterns with very strong anti-hatching activities (Fig. 1). Samples were grouped into two main unrelated clusters ($S_{corr} < 0.2$) (Fig. 1). Cluster I included EOs with specific volatile composition, namely those of *Filipendula ulmaria*, *Nepeta cataria*, *Ruta graveolens* and *Syzygium aromaticum* (Table 1). Cluster II grouped the remaining EOs and related HM and OCM fractions. This cluster grouped terpene-rich EOs and was sub-divided in several sub-clusters (Fig. 1).

Ineffective EOs, showing ≤10 % activity at the highest concentration (2 μL mL^{-1}), were dominated by e.g. the monoterpenes, α-pinene, sabinene, camphor and/or terpinen-4-ol (Table 1; Fig. 1). A total of 24 EOs were the most successful with a CHI ≥ 90 % at 2 μL mL^{-1}. Those isolated from *Dysphania ambrosioides* (isoascaridol 51 %, ascaridol 16 %), *Filipendula ulmaria* (methyl salicylate 85 %), *Foeniculum vulgare* 1 (*trans*-anethole 73 %) and 2 (methyl chavicol 79 %, limonene 12 %), *Genista tridentata* (*cis*-theaspirane 27 % and *trans*-theaspirane 22 %), *Mentha arvensis* (piperitenone oxide 56 %), *N. cataria* (4aα, 7α, 7aα-nepetalactone 89 %), *R. graveolens* (2-undecanone 91 %) and *S. aromaticum* (eugenol 92 %) had a low correlation with other EOs ($S_{corr} < 0.4$) (Fig. 1). Very strong inhibition percentages (≥90 %) at 2 μL mL^{-1} were also obtained for EOs showing highly correlated compositions ($S_{corr} > 0.7$). These were gathered in sub-clusters IIa ($S_{corr} > 0.7$), IIb ($S_{corr} > 0.9$), IId ($S_{corr} > 0.9$) and IIj ($S_{corr} > 0.8$) (Fig. 1). α-Terpineol (16–62 %), thymol (traces-23 %), linalool (traces-16 %)], terpinen-4-ol (1–16 %), γ-terpinene (traces-15 %), carvacrol (traces-15 %) and *p*-cymene (traces-13 %) dominated the EO composition of the samples grouped in the first cluster. Corrected hatching inhibitions ≥94 %, at 2 μL mL^{-1}, were obtained in sub-cluster IIb whose EOs showed thymol (32–45 %), *p*-cymene (14–22 %), γ-terpinene (6–16 %) and thymol acetate (0–15 %) as major components. Sub-cluster IId grouped samples with CHI ≥ 92 %, at 2 μL mL^{-1}, which revealed to be rich in carvacrol (45–96 %), *p*-cymene [not detected (nd)-22 %], γ-terpinene (nd-18 %) and carvacrol acetate (nd-14 %).

The highly hatching inhibitor *Cymbopogon citratus* EO grouped in sub-cluster IIj, with the corresponding OCM fraction, due to their richness in geranial (34–45 %), neral (22–36 %), β-myrcene (traces-20 %) and geraniol (5–18 %).

Very strong EOs that caused CHI ≥ 90 %, at 2 μL mL^{-1}, namely those of *D. ambrosioides*, *C. citratus*, *F. ulmaria*, *F. vulgare* 1, 2, *G. tridentata*, *M. arvensis*, *N. cataria*, *Origanum majorana, O. vulgare* subsp. *virens*, *O. vulgare*, *R. graveolens*, *Satureja montana* 1, 2, *S. aromaticum*, *Thymbra capitata*, *Th. caespititius* 2, 3, 4, 5, *Th. pulegioides*, *Th. vulgaris*, *Th. zygis* subsp. *silvestris* and *Th. zygis* subsp. *zygis* (Table 1; Fig. 1) were tested at lower concentrations. At the lowest concentration, 0.125 μL mL^{-1}, only the EOs extracted from *D. ambrosioides*, *F. ulmaria*, *R. graveolens*, *S. montana* 1, 2 and *T. capitata* exhibited a moderate to strong inhibitory activity. Half maximal effective concentrations (EC_{50}) for these EOs were calculated by fitting a dose–response log-logistic curve to the percentage CHI data. EC_{50} values obtained were 0.041 μL mL^{-1} for *D. ambrosioides*, 0.032 μL mL^{-1} for *Fi. ulmaria*, 0.061 and 0.033 μL mL^{-1} for *S. montana* 1 and 2, respectively, 0.121 μL mL^{-1} for *R. graveolens* and 0.140 μL mL^{-1} for *T. capitata* EOs (Table 2).

Hydrocarbons or oxygen-containing molecules fractions CRKN hatching inhibition

Due to their specific EO composition, a balanced percentage of hydrocarbons and OCM, and to CHI ≥ 90 %, the EOs of *C. citratus, O. vulgare, S. montana*, *T. capitata* and *Th. caespititius* 4 (Table 3) were chosen for fractionation, to evaluate the separate contribution of the HM and OCM fractions against hatching.

Oxygen-containing molecules fractions of these EOs revealed hatching inhibitions ≥92 % at 2 μL mL^{-1} (Fig. 1; Table 3). At the same concentration, the corresponding HM fractions showed activities ≤62 % (Fig. 1; Table 3).

The richness in oxygen compounds is reflected in cluster analysis (Fig. 1), as all OCM fractions grouped closely to their corresponding EOs ($S_{corr} \geq 0.8$). This was not observed for the HM fractions that grouped together ($S_{corr} \geq 0.7$), with the exception of *C. citratus*, showing similar chemical compositions (Fig. 1).

Given the results above, EC_{50} values were determined only for the most successful OCM fractions (Table 2), revealing additive/synergic interactions among the fractions. *S. montana* OCM fraction showed a higher EC_{50} value than that of the related EO, although the oxygen-containing compounds were present in higher proportions in the fraction. This suggests that in addition to the oxygen-containing compounds, the HM fraction also plays an important role in hatching toxicity of this EO. On the other hand, *Thymbra capitata* OCM fraction revealed a lower EC_{50} value than that of the related EO, suggesting that *M. chitwoodi* hatching toxicity may be EO specific.

Discussion

The present work is the first screening of EOs and EO fractions with hatching inhibition activity on the CRKN. *D. ambrosioides*, *F. ulmaria*, *R. graveolens*, *S. montana* and *T. capitata* EOs were herewith shown to have the lowest EC_{50} values against CRKN hatching. The main compounds of these EOs, namely 2-undecanone, ascaridol, carvacrol,

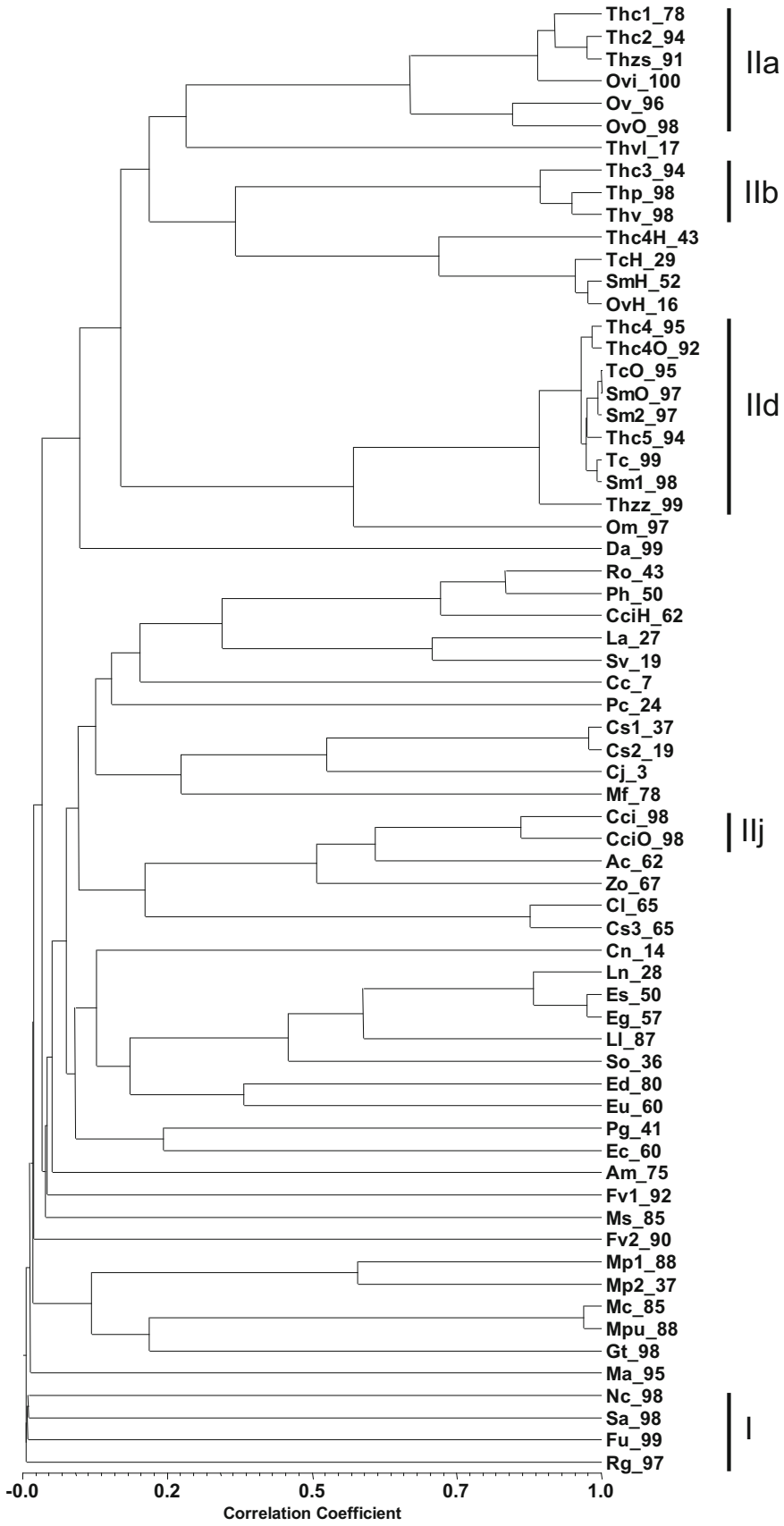

Fig. 1 Dendrogram obtained by cluster analysis of the full percentage composition of essential oils (EOs) from the 56 samples and 10 fractions based on correlation and using unweighted pair group method with arithmetic average. For each EO sample abbreviation, see Table 1. EO fractions abbreviations begin with the sample code followed by uppercase H for fractions containing HM or uppercase O for OCM. *Values* after underscore are the mean hatching inhibition percentages obtained with an EO concentration of 2 μL mL^{-1}

Table 2 EC_{50} values (μL mL^{-1}) of the most active essential oils (EOs) and related oxygen-containing molecules (OCM) fractions against *Meloidogyne chitwoodi* hatching

EOs/OCM	Code	EC_{50}	$Cl_{95\ \%}$	R^2
Dysphania ambrosioides	Da	0.041	0.016–0.108	0.94
Filipendula ulmaria	Fu	0.032	0.013–0.083	0.96
Ruta graveolens	Rg	0.121	0.107–0.136	0.99
Satureja montana 1	Sm1	0.061	0.028–0.133	0.94
Satureja montana 1 O	Sm1O	0.099	0.075–0.132	0.98
Satureja montana 2	Sm2	0.033	0.019–0.058	0.98
Thymbra capitata	Tc	0.140	0.125–0.157	0.99
Thymbra capitata O	TcO	0.120	0.114–0.126	0.99

O—EO oxygen-containing molecules fraction

The R^2 values and the 95 % confidence limits (Cl 95 %) are given for toxicity comparison

isoascaridol, methyl salicylate, *p*-cymene and/or γ-terpinene, were putatively considered responsible for CRKN hatching inhibition.

Ruta genus EOs nematotoxic activities have been previously described for other *Meloidogyne* species. *Ruta chalepensis* EO, also 2-undecanone-rich, displayed a high activity against *M. incognita* and *M. javanica* J2 motility (Ntalli et al. 2011b). Its mode of action is still unknown but aliphatic compounds are known to have high inhibition activities against acetylcholinesterase and glutathione S-transferase in the pinewood nematode (*B. xylophilus*) (Kang et al. 2013).

Thymus caespititius chemotypes, rich in carvacrol, thymol and α-terpineol, showed strong to very strong hatching inhibition, displaying CHI ≥ 78 %, at 2 μL mL^{-1}. Nevertheless, as described by Faria et al. (2013) studying nematotoxic EOs against pinewood nematode *B. xylophilus* motility, the occurrence of chemotypes must be taken into account when choosing a nematotoxic EO bearing-species, since EO particular chemotype may be determinant in this activity.

Methyl salicylate has shown strong nematicidal properties against *B. xylophilus*, both as a synthetic chemical (at 2 mg mL^{-1}) and as a major *Gaultheria fragrantissima* EO component (95 %) (at 5 mg mL^{-1}) (Kim et al. 2011). This compound is known to be emitted by stressed plants as a signal involved in eliciting plant resistance (Loake and Grant 2007). Pest management using this EO may take advantage of these characteristics by inhibiting hatching and also stimulating the plant immune response.

D. ambrosioides EO and its components were assessed against *M. incognita* revealing low LC_{50} values (Bai et al. 2011). The EO showed LC_{50} values ×20 lower than some of its main components, which indicates heavy additive or synergic compound relations.

Satureja montana 1, 2 and *T. capitata* EOs had similar volatile compositions, being carvacrol- (64, 77 and 68 %, respectively) and γ-terpinene-rich (18, 5 and 11 %, respectively). EOs rich in the oxygen-containing monoterpenoids carvacrol and thymol are known to have nematotoxic activity against plant parasitic nematodes (Oka et al. 2000; Kong et al. 2007; Barbosa et al. 2010, 2012; Faria et al. 2013). Anti-nematode activities of *S. montana* EOs have been demonstrated against hatching and J2 motility of *M. javanica* (Andrés et al. 2012), yielding similar results to the obtained in the present work.

Monoterpenoid activity against *M. incognita* hatching and J2 juvenile motility was tested, in vitro, by Echeverrigaray et al. (2010). Of the compounds tested, high nematotoxic activities were obtained for the oxygen-containing monoterpenes borneol, carveol, citral (mixture of geranial and neral), geraniol and α-terpineol. Oka et al. (2000) showed that the monoterpenes carvacrol, thymol and *trans*-anethole also revealed high activities against *M. javanica*

Table 3 Corrected hatching inhibition (CHI) percentages of the essential oils (EOs) and corresponding fractions, at 2 μL mL^{-1} (mean ± S.E., in %) and main components (≥10 %) of the EOs hydrocarbon molecules (HM) and oxygen-containing molecules (OCM) fractions

	CHI (%)			EOs fractions main composition (%)[a]	
Plant species[b]	EOs	HM	OCM	HM	OCM
Cymbopogon citratus	98 ± 1	62 ± 2	98 ± 1	β-Myrcene 72	Geranial 45, neral 36
Origanum vulgare	100 ± 0	33 ± 11	97 ± 3	γ-Terpinene 36, *p*-cymene 11	α-Terpineol 26, thymol 23, terpinen-4-ol 16, carvacrol 15, linalool 14
Satureja montana	98 ± 2	52 ± 6	97 ± 2	γ-Terpinene 44, *p*-cymene 19	Carvacrol 96
Thymbra capitata	99 ± 1	29 ± 5	95 ± 3	γ-Terpinene 36, *p*-cymene 23	Carvacrol 93
Thymus caespititius 4	95 ± 2	43 ± 2	92 ± 3	*p*-Cymene 29, γ-terpinene 16, *trans*-dehydroagarofuran 12	Carvacrol 66, carvacrol acetate 14

Values are means of ten replicates

[a] Fraction detailed composition in Faria et al. (2013)

[b] EOs chosen for fractionation showed hatching inhibition ≥90 % at 2 μL mL^{-1}

hatching and J2 juvenile motility. In the present study, high *M. chitwoodi* hatching inhibitions (≥90 %), at 2 μL mL^{-1}, were observed not only for the monoterpene-rich EOs of *Thymus caespititius* 2 and *T. zygis* subsp. *silvestris* (α-terpineol, 62 and 60 %, respectively), and *C. citratus* (geranial−34 %, neral−22 % and geraniol−18 %) but also for the *trans*-anethole-rich *Foeniculum vulgare* 1 (73 %). The activity of geraniol-, citronellol- and linalool-rich *Pelargonium graveolens* EO was also tested against *M. incognita* J2 motility in direct contact assays, being highly nematotoxic. Commercial EO compounds were evaluated individually, in the concentrations found in the EO, suggesting that the combined effect of the constituents also play a role in the EO nematicidal activity (Leela et al. 1992).

Synergistic action of basil (*Ocimum* spp.) EO components, methyl chavicol and linalool, was found against *Meloidogyne incognita*, *Heterodera avenae*, *H. cajani* and *H. zeae* (Gokte et al. 1991), while individually each compound showed no appreciable nematicidal activity.

Oxygen-containing molecules fractions appear to contribute deeply to the EO hatching inhibition. The same has been suggested by Abd-Elgawad and Omer (1995), analysing EO effects on phytoparasitic nematodes hatching (*Meloidogyne incognita*) and juvenile motility (*Rotylenchulus reniformis*, *Criconemella* spp., *Hoplolaimus* spp.). In the present study, the activity of separate components was not assessed; nevertheless, evaluation of the isolated HM and OCM fractions against *M. chitwoodi* hatching showed that, for the activity of an EO, all its components play a distinct role, contributing in more than one way, either synergistically or antagonistically. Ntalli et al. (2011a) analysed these types of interactions among the terpene components of EOs active against *M. incognita* showing that combinations of nematotoxic terpenes, such as carvacrol/thymol or carvacrol/geraniol, and/or phenylpropanoids, methyl chavicol/geraniol or *trans*-anethole, had a synergistic activity against J2 motility. This study did not include interactions among non-nematotoxic and nematotoxic EO components.

According to the present results, both the highly active oxygen-containing terpenes and the low hatching inhibition hydrocarbon terpenes cooperate against CRKN hatching. The higher activities of oxygen-containing monoterpenes against phytoparasitic nematodes has been described in several previous studies, but, to our knowledge, this is the first report on the hatching inhibition activity of EOs and their fractions against *M. chitwoodi*. Fractionation and evaluation of the fractions activities containing HM or OCM revealed that this approach, for some EOs, may improve hatching inhibition.

The high nematotoxic properties of some EOs encourage their use as environmentally safer nematicides for the management of the CRKN taken into account that the use of EOs is a highly complex method. Their use as pesticides must first be analysed in a host/parasite environment, since allelopathic effects can be evident when applying EOs to plant tissues. Direct in vitro assays must be complemented by in vivo, soil-based experiments, in order to examine phytotoxicity or plant biotransformation.

With a few exceptions, natural nematicides, like the 5 nematotoxic EOs presented herewith, are considered less hazardous than chemical synthetic nematicides (Figueiredo et al. 2008; Moharramipour and Negahban 2014). However, when considering environmentally friendly pest management tools for root-knot nematodes, management of high volatility of EOs through formulation, potential processing costs for large scale production, and the availability of plant biomass on a sustainable basis to meet agricultural needs should all be taken into consideration if commercialization is to be realized.

To better assess the infection mechanism and the plant response to nematotoxics, laboratory assays using host/parasite in vitro cultures that mimic as closely as possible the field environment are being conducted.

Author contribution statement

JMSF conceived, designed research, conducted the experiment and wrote the manuscript. IS, BR and AMR conducted the experiment. CMNM and IA supplied *M. chitwoodi* initial inoculum and reviewed the manuscript. RB, MM and ACF supervised the work and reviewed the manuscript. All authors read and approved the manuscript.

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