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Nematicidal activity of essential oils and volatiles derived from Portuguese aromatic flora against the pinewood nematode, *Bursaphelenchus xylophilus*

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Abstract: Twenty seven essential oils, isolated from plants representing 11 families of Portuguese flora, were screened for their nematicidal activity against the pinewood nematode (PWN), *Bursaphelenchus xylophilus*. The essential oils were isolated by hydrodistillation and the volatiles by distillation-extraction, and both were analysed by GC and GC-MS. High nematicidal activity was achieved with essential oils from *Chamaespartium tridentatum*, *Origanum vulgare*, *Satureja montana*, *Thymbra capitata*, and *Thymus caespititius*. All of these essential oils had an estimated minimum inhibitory concentration ranging between 0.097 and 0.374 mg/ml and a lethal concentration necessary to kill 100% of the population (LC_{100}) between 0.858 and 1.984 mg/ml. Good nematicidal activity was also obtained with the essential oil from *Cymbopogon citratus*. The dominant components of the effective oils were 1–octen-3-ol (9%), *n*–nonanal, and linalool (both 7%) in *C. tridentatum*, geranial (43%), neral (29%), and β-myrcene (25%) in *C. citratus*, carvacrol (36% and 39%), γ-terpinene (24% and 40%), and *p*-cymene (14% and 7%) in *O. vulgare* and *S. montana*, respectively, and carvacrol (75% and 65%, respectively) in *T. capitata* and *T. caespititius*. The other essential oils obtained from Portuguese flora yielded weak or no activity. Five essential oils with nematicidal activity against PWN are reported for the first time.

Key words: medicinal and aromatic plants, biological control, *Bursaphelenchus xylophilus*, essential oils, nematicidal activity, pinewood nematode, Portugal, volatiles, *Artemisia absinthium*, *Artemisia dracunculus*, *Calamintha baetica*, *Chamaespartium tridentatum*, *Chamomilla recutita*, *Cistus ladanifer*, *Crithmum maritimum*, *Cryptomeria japonica*, *Cymbopogon citratus*, *Foeniculum vulgare*, *Juniperus brevifolia*, *Laurus azorica*, *Laurus nobilis*, *Lavandula dentata*, *Lavandula luisieri*, *Lavandula stoechas*, *Lavandula viridis*, *Mentha pulegium*, *Myrtus communis*, *Origanum vulgare*, *Pittosporum undulatum*, *Salvia officinalis*, *Satureja montana*, *Thymbra capitata*, *Thymus caespititius*, *Thymus mastichina*, *Thymus zygis*.

Bursaphelenchus xylophilus (Steiner and Buhrer, 1934) Nickle, 1970 (pinewood nematode: PWN) the causal agent of pinewilt (PW), is associated with cerambycid beetles, particularly *Monochamus* spp. In forest ecosystems, PWN is considered one of the most important pathogens of coniferous forests worldwide (Mota and Vieira, 2008). The control of PW has been achieved mainly by controlling the insect vector by aerial application of synthetic insecticides, by fumigation of infected trees, or by controlling the nematode by trunk injection of anti-nematodal compounds (Lee et al., 2003; Takai et al., 2003). Unfortunately, many of the most effective chemicals used for controlling PW are highly toxic, very expensive, and can accumulate in the soil causing negative environmental impacts.

Recently, researchers have begun to look for alternative and equally effective natural products to replace current chemical control options (Chitwood, 2002; Rugutt et al., 2006). Because of European Union (EU) environmental restrictions, it has become necessary to develop new control techniques based upon natural products, which are perceived as safer for people and the environment. Essential oils, obtained from flowers or fruits of certain aromatic plants, have long been known to have significant biological activities (Duke, 1990; Isman, 2000; Prabuseenivasan et al., 2006). There are many reports on the preparation, chemical characterization, and biological effects of essential oils from a diversity of plant species (Kong et al., 2007; Takaishi et al., 2008). Studies have shown that some essential oils have good nematicidal activities (Oka, 2001; Pérez et al., 2003). The majority of studies evaluating PWN control based on essential oils have been developed in countries affected by this nematode, such as China (Hong et al., 2007), Japan (Alen et al., 2000), and South Korea (Choi et al., 2007). Under laboratory conditions, essential oils extracted from *Myristica malabarica* (Choi et al., 2008), *Brassica integrefolia*, *Pelargonium inquinans*, *Thymus vulgaris* (Elbadri et al., 2008), and *Coriandrum sativum*, *Liquidambar orientalis* and *Valeriana wallichii* (Kim et al., 2008) had significant activity against PWN. Presently, the increasing dispersion of PWN in Portugal (MADRP, 2008), and more recently in Spain, has lead to new lines of research focusing on developing new methods to control PW.

The objective of this research was the identification and selection of essential oils with high nematicidal activity against PWN for further fractionation and bioassay-guided isolation of active compounds. Tested plants were selected from amongst Portuguese flora, with special interest given to the most common aromatic plants.

Material and Methods

Plant material: The aerial parts of several Portuguese flora species, from collective or individual samples,

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were collected from wild-growing plants on the mainland of Portugal and in the Azores archipelago (Portugal) (Table 1). Plant material was stored at -20°C until extraction. Dried aerial parts from commercially available products (phytoceuticals) sold in local herbal shops were also evaluated (Table 1). In total essential oils from 27 taxa representing 11 families were evaluated for nematicidal activity and chemical composition.

Essential oil and volatile extraction: Essential oils were isolated by hydrodistillation (H) for 3 hr using a Clevenger-type apparatus according to the European Pharmacopoeia method (Council of Europe, 2007) (Table 2). Volatiles were isolated by distillation-extraction (D–E) for 3 hr using a Likens-Nickerson-type apparatus (Likens and Nickerson, 1964) with distilled *n*–pentane (50 ml) (Honeywell Riedel-de Haën, Hanover, Germany) as the organic solvent (Table 2). Both isolation procedures were run at a distillation rate of 3 ml/min and, on average, at least 100 g of each plant was extracted. The D-E oils recovered in distilled *n*–pentane were concentrated at room temperature under reduced pressure on a rotary evaporator, collected in a vial, and concentrated to a minimum volume, again at room temperature, under nitrogen flux. Essential oils and volatiles were stored in the dark at -20°C until analysis.

Gas Chromatography (GC): Gas chromatographic analyses were performed using a Perkin Elmer Autosystem XL chromatograph (Perkin Elmer, Shelton, CT) equipped with two flame ionization detectors (FIDs), a data handling system, and a vaporizing injector port into which two columns of different polarities were installed: a DB-1 fused-silica column (30 m x 0.25 mm i.d., film thickness 0.25 μm; J & W Scientific Inc., Rancho Cordova, CA) and a DB-17HT fused-silica column (30 m x 0.25 mm i.d., film thickness 0.15 μm; J & W Scientific Inc.). Oven temperature was programmed to increase from 45 to 175°C, at 3°C/min increments, then up to 300°C at 15°C/min increments, and finally held isothermal for 10 min. Gas chromatographic settings were as follows: injector and detector temperatures, 280°C and 300°C, respectively; carrier gas, hydrogen, adjusted to a linear velocity of 30 cm/s. 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 oil, without response factors.

Gas Chromatography - Mass Spectrometry (GC-MS): The GC-MS unit consisted of a Perkin Elmer Autosystem XL chromatograph, equipped with DB-1 fused-silica column (30mx0.25mm i.d., film thickness 0.25 μm; J & W Scientific, Inc.) 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; split ratio, 1:40; ionization energy, 70eV; scan range, 40-300 u; scan time, 1 s. The identity of the

Table 1. Sampling dates, sources, and families of the taxa tested for essential oils and volatiles nematicidal activity.

Species	Family	Collection place or source	Sampling date
Artemisia absinthium L.	Asteraceae	Évora	2007 and 2009
Artemisia dracunculus L.	Asteraceae	Évora	2007
Calamintha baetica Boiss. & Reut.	Lamiaceae	Évora	2008
Chamaespartium tridentatum (L.) P. Gibbs	Fabaceae	Herbal shop	2008
Chamomilla recutita (L.) Rauschert	Asteraceae	Herbal shop	2008
Cistus ladanifer L.	Cistaceae	Faro	2008
Crithmum maritimum L.	Apiaceae	Lisbon	2007
Cryptomeria japonica (L. fil.) D. Don.	Taxodiaceae	São Jorge, Azores	2008
Cymbopogon citratus (DC) Stapf.	Poaceae	Faro	2008
Foeniculum vulgare Miller	Apiaceae	Évora	2007
Juniperus brevifolia (Seub.) Antoine	Cupressaceae	Faial, Azores	2008
Laurus azorica (Seub.) J. Franco	Lauraceae	São Miguel, Azores	2008
Laurus nobilis L.	Lauraceae	Herbal shop	2008
Lavandula dentata L.	Lamiaceae	Lisbon	2008
Lavandula luisieri (Rozeira) Rivas Martinez	Lamiaceae	Faro	2008
Lavandula stoechas L.	Lamiaceae	Faro	2008
Lavandula viridis L'Hér.	Lamiaceae	Faro	2008
Mentha pulegium L.	Lamiaceae	Évora	2007
Myrtus communis L.	Myrtaceae	Évora	2007
Origanum vulgare L.	Lamiaceae	Évora	2007
Pittosporum undulatum Vent.	Pittosporaceae	Lisbon	2007
Salvia officinalis L.	Lamiaceae	Évora	2007
Satureja montana L.	Lamiaceae	Évora	2008
Thymbra capitata (L.) Cav.	Lamiaceae	Faro	2008
Thymus caespititius Brot.	Lamiaceae	São Miguel, Azores	2008
Thymus mastichina L.	Lamiaceae	Santarém	2008
Thymus zygis L.	Lamiaceae	Faro	2008

TABLE 2. Nematicidal activity of 27 essential oils against *Bursaphelenchus xylophilus* exposed for 24 hr to a 2 mg/ml solution.

Species	Plant part	Status	I.P.[a]	Oil Yield (%, v/w)	Mortality (%)[b]
Artemisia absinthium L.	Aerial parts in vegetative phase	Fresh	H	0.20	-0.49 ± 0.52
Artemisia dracunculus L.	Aerial parts in vegetative phase	Fresh	H	1.50	22.88 ± 2.51*
Calamintha baetica Boiss. & Reut.	Aerial parts in vegetative phase	Dried	H	2.80	1.91 ± 3.29
Chamaespartium tridentatum (L.) P. Gibbs	Flowers	Dried	H	0.01	100 ± 0*
Chamomilla recutita (L.) Rauschert	Flowers	Dried	H	0.13	8.82 ± 2.82
Cistus ladanifer L.	Aerial parts in vegetative phase	Fresh	D-E		2.49 ± 0.76
Crithmum maritimum L.	Aerial parts in vegetative phase	Fresh	H	0.20	2.05 ± 1.02
Cryptomeria japonica (L. fil.) D. Don.	Mature cones and berries	Fresh	D-E		0.23 ± 0.96
Cymbopogon citratus (DC) Stapf.	Leaves	Fresh	H	0.80	98.12 ± 0.30*
Foeniculum vulgare Miller	Aerial parts in vegetative phase	Fresh	H	0.50	19.82 ± 2.12*
Juniperus brevifolia (Seub.) Antoine	Aerial parts in vegetative phase	Fresh	D-E		-2.62 ± 0.56*
Laurus azorica (Seub.) J. Franco	Aerial parts in vegetative phase	Fresh	D-E		2.17 ± 0.53
Laurus nobilis L.	Leaves	Dried	H	1.30	1.37 ± 0.57
Lavandula dentata L.	Aerial parts in flowering phase	Fresh	H	0.20	-0.28 ± 0.54
Lavandula luisieri (Rozeira) Rivas Martinez	Aerial parts in flowering phase	Fresh	D-E		0.55 ± 0.79
Lavandula stoechas L.	Aerial parts in flowering phase	Fresh	D-E		3.45 ± 0.68
Lavandula viridis L'Hér.	Aerial parts in flowering phase	Fresh	D-E		3.59 ± 1.20
Mentha pulegium L.	Aerial parts in flowering phase	Fresh	H	2.00	68.60 ± 1.77*
Myrtus communis L.	Aerial parts in flowering phase	Fresh	H	0.20	10.87 ± 2.22*
Origanum vulgare L.	Aerial parts in flowering phase	Fresh	H	1.70	100 ± 0*
Pittosporum undulatum Vent.	Aerial parts in vegetative phase	Fresh	H	0.05	0.65 ± 0.52
Salvia officinalis L.	Aerial parts in flowering phase	Fresh	H	0.60	0.23 ± 0.45
Satureja montana L.	Leaves	Dried	H	1.60	100 ± 0*
Thymbra capitata (L.) Cav.	Aerial parts in flowering phase	Fresh	D-E		100 ± 0*
Thymus caespititius Brot.	Aerial parts in flowering phase	Fresh	D-E		100 ± 0*
Thymus mastichina L.	Aerial parts in flowering phase	Fresh	D-E		0.56 ± 0.56
Thymus zygis L.	Aerial parts in flowering phase	Fresh	D-E		86.59 ± 3.94*

[a] I.P= isolation procedure. For each species isolation was either by hydrodistillation (H) or distillation-extraction (D-E).
[b] Mortality values with * have 99% confidence intervals not including zero. *C. ladanifer, L. azorica, L. nobilis, L. dentata, L. luisieri, L. stoechas, L. viridis*, and *T. mastichina* with n = 3; all other species n = 5.

components was assigned by comparison of their retention indices relative to C_8-C_{21} *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.

Nematodes rearing and collection: Wood chips from a maritime pine (*Pinus pinaster*) tree displaying wilt symptoms were collected in the Setúbal region, Portugal. Collected PWN were maintained in Petri dishes containing *Botrytis cinerea* cultured on malt extract agar. Prior to testing, cultured nematodes were separated from the agar medium for 48 hr in a Baermann tray, placed in a new fungal culture, and left to multiply for one week at 25°C in the dark. Nematodes were separated from the culture medium as described above and counted under a binocular microscope Olympus SZX-12 (Olympus Corporation, Tokyo, Japan). A nematode suspension in distilled water was made by a series of dilutions, such that 100 µl contained between 100 and 200 mixed-stage nematodes.

Bioassays: Bioassays were performed in a 96-well microtiter plates (Carl Roth GmbH + Co. KG, Karlsruhe, Germany). In each well, the essential oil solution (1 µl) was added, followed by the nematode suspension (99 µl). Concentrations were prepared once and five wells were used per concentration and essential oil, except in eight species were n=3 (Table 1). Triton X-100 (Scharlau Chemie, Barcelona, Spain) in distilled water solution (5 g/ml) was included as the control. The plate was placed in a vortex apparatus at 500 RPM for 2 min and stored at 25°C in the dark. After 24 hr, dead and live nematodes were counted under a binocular scope (Olympus SZX-12). Nematodes were considered dead if they did not move even when mechanical stimulated. The first tested concentration of each essential oil was 2 mg/ml, prepared from the pure oil extract and the Triton X-100 solution (5 g/ml). A series of dilutions from the Triton X-100 solution, 1 mg/ml, 0.5 mg/ml, 0.25 mg/ml and 0.125 mg/ml, were made and tested when the mortality at 2 mg/ml was greater than 90%.

Data analysis: To account for the observed mortality in the controls (M_0), mortality in the treatments (M_T) was corrected as mortality corrected (M_C) = $M_T - M_0 / 100 - M_0$ (B. G. Zhao, Nanjing Forest University, personal communication). Confidence intervals of 99% for M_C were used to identify essential oils active against PWN.

The relation between M_C and concentration was investigated by fitting the Weibull function (Weibull, 1951) by least squares nonlinear regression without replication using the Marquardt method (Marquardt, 1963). The three parameter Weibull function is expressed as

$$M_C = 1 - \exp - \{[(X - l)/k]^c\}$$

where M_C is the observed corrected mortality (in proportion) at essential oil concentration X, l is a location

parameter, k a scale parameter, and c a shape parameter. When mortality at the highest concentration was less than 100% the Weibull function fails to asymptote in the range of concentrations tested and a fourth parameter, M_{max}, was added to the Weibull function as described in Brown (1987). The l parameter of Weibull function represents the highest concentration of essential oil at which the mortality is strictly zero (Bonner and Dell, 1976). For all practical purposes l values represent the minimum concentration effective against PWN and can be used to compare and rank the activity of essential oils at low concentrations. The k parameter of the Weibull function represents the concentration at which the mortality is approximately 63% when $l = 0$. Therefore $l + k$ estimate the concentration at which PWN mortality is approximately 63% (Bonner and Dell, 1976). In most dose response studies, estimating LC_{63} would be more than enough. However, to control PWN effectively, LC_{63} is clearly a less than desirable target, with 100% mortality desired and therefore values of LC_{100} were calculated from fitted equations. The c parameter evaluates the symmetry of the distribution with Weibull functions with $3.25 \leq c \leq 3.61$ showing symmetry and representing a good approximation to the normal distribution, $c < 3.25$ positive, $c > 3.61$ negative asymmetry (Bonner and Dell, 1976; Dubey, 1967). Symmetry is known to be generated by factors acting additively, positive asymmetry can be easily generated by factors acting multiplicatively (Limpert et al., 2001) while negative asymmetry can be generated by fraction powers. The M_{max} parameter estimates the maximum nematode mortality given the overall dose response curve.

Replicates were defined by their rank of corrected mortality and fitted equations were only accepted after a consistency check of parameter estimates and mortality predictions against original data. The effects of essential oils on l, k, c, and LC_{100} were compared using a least squares linear regression approach with dummy variables to prevent the occurrence of lack of "transitivity" (Chew, 1976; Penas et al., 2002). Forward stepwise selection with replication was used and the candidate model included qualitative variables only, namely the species source of the essential oil (coded as 1, 0), with an experiment-wise Type I error rate of 0.01 for coefficients calculated using the Dunn-Šidák method (Sokal and Rohlf, 1995). Results of bioassays are presented as means ± standard errors.

Results

Nematicidal activity: Initially the toxic effect of 27 essential oils was evaluated at 2 mg/ml (Table 2). After 24 hr, mortality of nematodes was significantly higher than 90% with essential oils obtained from *C. citratus*, *C. tridendatum*, *O. vulgare*, *S. montana*, *T. capitata*, and *T. caespititius*. In 12 out of the 27 species tested, the 99% confidence interval of mean mortality at 2 mg/ml did not include zero mortality and therefore significant effects of essential oils on PWN occurred (Table 2). Increases in nematode mortality were almost always found when the effects of essential oils at 2 mg/ml were significant. The exception was the essential oil of *J. brevifolia* that significantly reduced mortality of PWN.

The three or four term Weibull function could always be fitted to the effects of essential oils of *C. citratus*, *C. tridendatum*, *O. vulgare*, *S. montana*, *T. capitata* and *T. caespititius* on PWN mortality. Coefficients of determination were always greater than 0.875 (0.980 ± 0.006). Estimated values of Weibull coefficients and LC_{100} are summarized in Table 3 together with significant differences between essential oil effects for an experiment-wise error rate of 0.01.

Origanum vulgare contained the most active essential oil at low dosage given its mean l value of 0.023 ± 0.008 mg/ml. The l values for the second most active essential oil (*T. capitata*), was more than four times higher (0.097 ± 0.001 mg/ml) than that of *O. vulgare*. No significant differences were found between l values of *C. citratus* and *S. montana*, with the least effective essential oil being isolated from *C. tridentatum* with an l value about 16 times higher (0.374 ± 0.004 mg/ml) than *O. vulgare*.

Among the six plant species bioassayed at a range of concentrations, only *C. citratus* did not result in 100% mortality with an estimated M_{max} of 99.38 ± 0.17% at a relatively high concentration (2.870 ± 0.150 mg/ml). Essential oils from the remaining five plant species caused

Table 3. Estimated values of Weibull coefficients (l, k, c)[a] and lethal concentration values necessary to result in 100% *Bursaphelenchus xylophilus* mortality (LC_{100}).

Species	l	k	c	LC_{100}
Chamaespartium tridentatum	0.374 ± 0.004 a[b]	0.778 ± 0.006 a	2.797 ± 0.031a	1.981 ± 0.004 a
Cymbopogon citratus	0.145 ± 0.020 b	0.272 ± 0.038 b	1.180 ± 0.090 b	[c]
Origanum vulgare	0.023 ± 0.008 c	1.284 ± 0.012 c	4.797 ± 0.109 c	1.984 ± 0.008 b
Satureja montana	0.142 ± 0.010 b	0.279 ± 0.020 b	2.310 ± 0.064 a	0.858 ± 0.062 c
Thymbra capitata	0.097 ± 0.001 d	0.457 ± 0.025 d	3.083 ± 0.199 a	0.985 ± 0.009 d
Thymus caespititius	0.218 ± 0.003 e	0.210 ± 0.003 b	1.783 ± 0.033 d	0.874 ± 0.007 e

[a] $M_C = 1 - \exp - \{ [(X - l)/ k]^c \}$ where M_C is the corrected mortality at essential oil concentration X, l is the highest concentration of essential oil at which the mortality is strictly zero, k is the concentration of essential oil at which the mortality is approximately 63% when $l = 0$, and c evaluates the symmetry of mortality distribution.

[b] In each column, means with the same letter do not differ for an experiment-wise error rate of 0.01.

[c] Estimated maximum mortality M_{max} = 99.38±0.17%. $M_C = M_{max} (1 - \exp - \{ [(X - l)/ k]^c \})$ where M_C, X, l, k and c as above, M_{max} estimates the maximum mortality for the dose response curve.

nematode mortality with the estimated concentrations for LC_{100} of *S. montana* and *T. caespititius* not significantly different. The essential oils of *S. montana* and *T. caespititius* were the most toxic, with the lowest LC_{100} (0.858 ± 0.062 mg/ml and 0.874 ± 0.007 mg/ml, respectively) followed by *T. capitata* (0.985 ± 0.009 mg/ml). The most effective essential oil identified by the parameter *l*, *O. vulgare*, had the highest LC_{100} (1.984 ± 0.008 mg/ml).

The essential oil of *C. citratus* had the lowest *c* value (1.180 ± 0.090), followed by *T. caespititius* (1.783 ± 0.033), both highly positively asymmetric, while *O. vulgare* had the highest *c* value (4.797 ± 0.109), negatively asymmetric. Essential oils of the remaining species had *c* values not significantly different from each other and close to the lower limit of *c* for a symmetric distribution.

Essential oil composition: Of the 27 essential oils isolated and chemically characterized from Portuguese flora, only those of *C. tridentatum*, *C. citratus*, *O. vulgare*, *S. montana*, *T. capitata* and *T. caespititius*, are detailed in Table 4, since they were the only ones that demonstrated nematicidal activity. *Chamaespartium tridentatum* volatiles were dominated by a fraction termed "Others" (Table 4), because components that were neither terpenes nor phenylpropanoids are included. This fraction was mainly composed of non-aromatic alcohols, saturated and unsaturated non-aromatic aldehydes, hydrocarbons, and fatty acids (43%).

Monoterpenes dominated the oils isolated from the species in the family Lamiaceae, *O. vulgare* (95%), *S. montana* (99%), *T. capitata* (98%) and *T. caespititius* (92%). Carvacrol (36% and 39%), γ–terpinene (25% and 40%), and *p*-cymene (14% and 7%) were the main components of *O. vulgare* and *S. montana* oils, respectively. Carvacrol was also the main component in *T. capitata* (75%) and *T. caespititius* (65%) oils. *Cymbopogon citratus* oil (Poaceae), which also showed a good nematicidal activity, was dominated by geranial (43%), neral (29%) and β-myrcene (25%).

Discussion

Significant PWN mortality was achieved by essential oils of 11 Portuguese aromatic flora investigated at a concentration of 2 mg/ml. To our knowledge this is the first report of nematicidal activity against PWN by *C. tridentatum*, *S. montana*, *T. capitata*, *T. caespititius*, and *T. zygis*. Exposure of PWN to essential oils of six of the tested species produced more than 90% mortality. All of these species are angiosperms belonging to three different families with members of the Lamiaceae being the most common. Conversely, essential oils from the two gymnosperms tested failed to result in nematode mortality.

Using the Weibull model, *l* was useful to rank essential oils by their minimum active concentration and identify promising sources of compounds lethal to PWN at low dosages. However, *l* values fail to provide a basis to pinpoint essential oils with the ability to kill a sizeable fraction of PWN. According to their LC_{100} values, the essential oils of *S. montana*, *T. caespititius* and *T. capitata* are clearly those to select for fractionation and bioassay-guided search of highly active compounds able to effectively control PWN.

Compared to other parameters, values of *c* parameter of fitted Weibull functions might provide a more informed decision about the essential oils to select for further studies. In this study, complex mixtures of compounds were investigated for their nematicidal activity but the ultimate goal is to find one, or at the most a few chemicals, with the ability to kill PWN at the lowest possible concentration. Therefore, essential oils for which interaction of effects can be predicted are better choices for fractionation and bioassay-guided isolation of active compounds than essential oils with constituents acting additively. According to this reasoning, *C. citratus* would rank first for fractionation and bioassay-guided studies, followed by the essential oil of *T. caespititius* and by *O. vulgare*. All three should be selected considering the shape of their nematicidal activity. However, *C. citratus* produces an essential oil that did not kill all nematodes and therefore is less than ideal. The essential oil of *T. caespititius* had the second lowest LC_{100} while the essential oil of *O. vulgare* had the highest LC_{100} rendering it less attractive. Essential oils of the remaining species, including the species with the lowest LC_{100} (*S. montana*), had *c* values implying approximately a symmetric distribution of PWN mortality and in all likelihood absence of interactive effects.

Combining LC_{100} with *c* estimates, the essential oil of the labiate *T. caespititius* is the first choice for fractionation and bioassay-guided search of compounds able to completely control PWN. The grass *C. citratus* and the labiate *O. vulgare* could also be considered for further research provided that the predicted interaction of effects of the components of their essential oils are antagonistic, meaning that the activity of chemicals tested separately would be higher than their combined activity in essential oil.

Chamaespartium tridentatum essential oils are known to demonstrate major chemical variability as shown in the study of Grosso et al. (2007). Although some qualitative and quantitative differences can be found between the essential oil analysed in the present study and that of Grosso et al. (2007), both studies have in common that a fraction designated as "others" dominated the volatile oils. The volatile profiles of the four Lamiaceae species reported herewith as having nematicidal activity, were in accordance with previous studies on *O. vulgare* (Faleiro et al., 2005; Prieto et al., 2007), *S. montana* (Prieto et al., 2007, Grosso et al., 2009), *T. capitata* (Faleiro et al., 2005; Figueiredo et al., 2008a) and *T. caespititius* (Figueiredo et al., 2008a). Likewise, the essential oil composition of *C. citratus* was in agreement to that reported for the same species (Baratta et al., 1998; Nguefack et al., 2009).

TABLE 4. Chemical composition of the essential oils and volatiles of Portuguese plants nematicidal to *Bursaphelenchus xylophilus*. *Chamaespartium tridentatum (Ct)*, *Cymbopogon citratus (Cc)*, *Origanum vulgare (Ov)*, *Satureja montana (Sm)*, *Thymbra capitata (Tc)*, and *Thymus caespititius (Thc)*.

Compounds	RI[a]	Fabaceae	Lamiaceae				Poaceae
		Ct	*Ov*	*Sm*	*Tc*	*Thc*	*Cc*
trans-2-Hexenal	866	0.1					
n-Heptanal	897	0.9					
Tricyclene	921					t[b]	
α-Thujene	924		2.6	2.3	1.6	3.4	
Benzaldehyde	927	0.3					
α-Pinene	930	0.3	1.2	1.5	1.0	1.2	t
Camphene	938		0.2	0.1	0.1	0.1	t
Thuja-2,4(10)-diene[c]	940		t				
Sabinene	958		0.1		t	t	
6-Methyl-5-hepten-2-one	960						t
1-Octen-3-ol	961	9.2	0.3	0.3	0.2	t	
β-Pinene	963		0.5	0.5	0.1	0.2	
2-Pentyl furan	973	0.8					
n-Octanal	973	0.6					
Dehydro-1,8-cineole	973						t
3-Octanol	974		1.0	t	t		
β-Myrcene	975		1.9	2.5	2.4		24.7
α-Phellandrene	995		0.4	0.4	0.3	0.1	
δ-3-Carene	1000		0.1	0.1	0.1		
Benzyl alcohol	1000				t		
α-Terpinene	1002		3.1	4.1	1.8	0.6	
p-Cymene	1003	0.3	13.8	7.1	5.6	5.1	t
1,8-Cineole	1005	0.7			t		t
β-Phellandrene	1005		0.2	0.2	0.3	0.2	
Limonene	1009	t	0.5	0.4	0.3	0.2	t
cis-β-Ocimene	1017		0.7		t		0.4
trans-β-Ocimene	1027		0.1	t	t		0.1
γ-Terpinene	1035		23.5	39.8	6.7	3.2	
trans-Sabinene hydrate	1037		t	0.6	0.3	0.2	
n-Octanol	1045	0.7	t				
Fenchone	1050	t	t				
p-Cymenene	1050		t				
Heptanoic acid	1056	0.6					
2-Nonanone	1058						t
2,5-Dimethyl styrene	1059				t		
Terpinolene	1064		t	t	0.2	0.1	
cis-Sabinene hydrate	1066		t	0.1	0.1		
n-Nonanal	1073	6.5				t	
Linalool	1074	7.1	0.1		0.8	t	0.7
Perillene[a]	1076						t
cis-Rose oxide	1083	t					
1-Octen-3-ol acetate	1086					t	
trans-*p*-2-Menthen-1-ol	1095		t		t		
Camphor	1095	0.7					
trans-Pinocarveol	1106	0.3					
cis-Verbenol	1110						0.5
allo-Ocimene	1110		t				t
cis-*p*-2-Menthen-1-ol	1110				t		
2-*trans*-6-*cis*-Nonadienal	1110	0.2					
trans-Verbenol[a]	1114						1.0
Menthone	1120	0.2					
Benzyl acetate	1123	0.2					
Nerol oxide	1127	t					
Borneol	1134	1.1	0.2	0.1	0.1	0.1	
Lavandulol	1142	0.3					
Menthol	1148	0.5					
Terpinen-4-ol	1148	0.7	0.5	0.4	0.6	0.5	
p-Cymen-8-ol	1148				t		
Octanoic acid	1159	0.5					
cis-Dihydrocarvone	1159			t			
α-Terpineol	1159	1.8	t	t	0.1		

(*Continued*)

TABLE 4. Continued.

Compounds	RI[a]	Fabaceae	Lamiaceae				Poaceae
		Ct	*Ov*	*Sm*	*Tc*	*Thc*	*Cc*
Methyl chavicol (= estragole)	1163	0.9	t				
n-Decanal	1180	0.4					
Carvone	1206		t		t		
Thymol methyl ether	1208		t				
Pulegone	1210	1.4					
Neral	1210						28.6
Piperitone	1211						t
Carvacrol methyl ether	1224		7.9	0.2			
Phenyl ethyl acetate	1228	t					
Geraniol	1236	0.6					0.5
Geranial	1240				t		42.5
Linalyl acetate	1245	1.4					
trans-Anethole	1254	4.7	t				
n-Decanol	1259	0.6					
Nonanoic acid	1273	1.5					
2-Undecanone	1273	2.2					0.4
Thymol	1275		1.2	t	0.2		
Carvacrol	1286	0.3	35.7	38.8	75.0	65.3	
cis-Theaspirane	1286	3.2					
2-*trans*-4-*trans*-Decadienal	1286	1.0					
trans-Theaspirane	1300	3.9					
Hexyl tiglate ester	1316	0.2					
Eugenol	1327	0.8					
Thymol acetate	1330					0.5	
α-Terpenyl acetate	1334	0.3					
α-Longipinene	1338	0.1					
α-Cubebene	1345		t				
Carvacrol acetate	1348				0.1	10.8	
Decanoic acid	1350	0.8					
trans-β-Damascenone[c]	1356	0.8					
Geranyl acetate	1370	0.5					t
α-Copaene	1375		t				
β-Bourbonene	1379		t			0.1	
β-Elemene	1388		t				
2-Pentadecanone	1390	0.8					
α-Gurjunene	1400					t	
β-Caryophyllene	1414	1.2	1.9	0.5	1.4		t
β-Copaene	1426		t				
Geranyl ketone[c]	1434	0.7					
trans-α-Bergamotene	1434		t		t		t
Sesquisabinene[c]	1438						t
α-Humulene	1447		0.3		t		t
allo-Aromadendrene	1456	0.7				0.1	
trans-β-Ionone[c]	1456	1.1					
γ-Muurolene	1469		t				
Germacrene-D	1474		1.0			0.1	
α-Curcumene	1475	0.5					
cis-β-Farnesene	1476				t		t
trans-Muurola-4(14),5-diene[a]	1479		t				
Viridiflorene	1487		t				
Dodecanol allyl ether	1488						0.2
trans-β-Dihydroagarofuran[c]	1489					1.3	
α-Muurolene	1494					0.2	
β-Bisabolene	1500		0.8	t	0.2		
trans-Calamenene	1505					0.1	
δ-Cadinene	1505		t			0.6	
Kessane	1517					t	
Elemol	1530					t	
trans-α-Bisabolene	1536				0.2		
Geranyl butyrate	1544						t
Dodecanoic acid	1551	5.3					
β-Caryophyllene oxide	1561		t		0.1		t
Geranyl isovalerate	1590						t

(*Continued*)

Table 4. Continued.

Compounds	RI[a]	Fabaceae	Lamiaceae				Poaceae
		Ct	*Ov*	*Sm*	*Tc*	*Thc*	*Cc*
10-*epi*-γ-Eudesmol	1593						0.1
γ-Eudesmol	1609					t	
T-Cadinol	1616					0.1	
δ-Cadinol (=α-Muurolol)	1618					t	
β-Eudesmol	1620					t	
Intermedeol	1626		0.1				t
α-Eudesmol	1634					1.1	
Tetradecanoic acid	1734	0.2					
Hexadecanoic acid	1779	0.7					
9,12-Octadecadienoic acid	1820	0.4					
Rosadiene[a]	1993				0.1		
Abietatriene	2027				t		
% of identification		71.8	99.9	99.9	99.9	95.6	99.7
Grouped components							
Monoterpene hydrocarbons		0.6	48.9	59.0	20.5	14.3	25.2
Oxygen-containing monoterpenes		18.6	45.6	40.2	77.3	77.5	73.8
Sesquiterpene hydrocarbons		2.5	4.0	0.5	1.8	1.2	t
Oxygen-containing sesquiterpenes		7.1	0.2		0.1	2.6	0.1
Diterpenes					0.1		
Phenylpropanoids		6.4	t				
Fatty acids							
Others		36.6	1.3	03	0.2	t	0.6

[a] RI = Retention index relative to C_8-C_{21} *n*-alkanes on the DB1 column.
[b] t = trace (<0.05%);
[c] Identification based on mass spectra only.

Phenol, alcohol, and aldehyde compounds could be the most nematotoxic against PWN (Choi et al., 2007). Thymol and carvacrol are two such compounds with confirmed toxicity against PWN (Kong et al., 2007), *Caenorhabditis elegans*, and *Ascaris suum* (Lei et al., 2010). According to Nguefack et al. (2009), the biological activity of an essential oil against mycotoxigenic fungi was a function of the proportion in oxygenated monoterpenes and certain monoterpene hydrocarbons such as γ–terpinene and *p*-cymene. Lambert et al. (2001) reported that essential oil modes-of-action were due to damage to bacteria membrane integrity, affecting pH cellular homeostasis and equilibrium of inorganic ions. Although not evaluated in the present work, it is noteworthy to mention that a recent study by Lei et al. (2010) demonstrated that the nematicidal activity of thymol and carvacrol might be mediated through tyramine receptor (TyrR), as the two compounds were able to trigger a signalling cascade that lead to nematode mortality by interacting with a receptor like SER-2.

Oils from *A. absinthium*, *A. dracunculus*, *C. citratus*, *M. pulegium*, *M. communis*, *O. vulgare*, *S. officinalis*, and *T. mastichina* were previously assessed for nematicidal potential (Kong et al., 2006). *Cymbopogon citratus* oil was also evaluated by Park et al. (2005) and in a separate study was reported to have nematicidal activity against *Meloidogyne javanica* (Oka et al., 2000). *Origanum vulgare* and *T. mastichina* essential oils showed nematicidal effect against *Ditylenchus dipsaci* (Zouhar et al., 2009).

Our results are similar to those previously obtained, except for *M. pulegium*. At a concentration of 2 mg/ml *M. pulegium* oil approximately 60% of PWN were killed; while at a higher concentration (10 mg/ml) no nematicidal activity was reported by Kong et al. (2006). Variability in essential oil composition and yield is known to occur, namely due to physiological variation, environmental conditions, and geographic variation (Figueiredo et al., 2008b). Chemical variability is responsible for the existence of oil chemotypes which may partly explain the dissimilarities in the properties of the oils isolated from the same species. Results from this study indicate that essential oils from medicinal and aromatic plants from Portuguese flora could be used to control PWN populations. Additional studies will be necessary to identify the most effective components from each oil and to develop more effective formulas with low production costs.

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