Nematicidal Activity of the Essential Oil of *Rhododendron anthopogonoides* Aerial Parts and its Constituent Compounds against *Meloidogyne incognita*

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Hydrodistilled essential oil from *Rhododendron anthopogonoides* Maxim. (Ericaceae) aerial parts was analysed by gas chromatography-mass spectrometry (GC-MS). A total of 42 compounds, accounting for 95.48% of the total oil, were identified. The main constituents of the essential oil were benzyl acetone (34.41%), nerolidol (10.19%), 1,4-cineole (8.41%), β-caryophyllene (5.63%), γ-elemene (5.10%), and spathulenol (3.06%). Four constituents were isolated from the essential oil based on fractionation. The essential oil of *R. anthopogonoides* possessed nematicidal activity against the root knot nematode (*Meloidogyne incognita*) with an LC_{50} value of 130.11 μg/ml. The main compound of the essential oil, benzyl acetone, exhibited nematicidal activity against *M. incognita* with an LC_{50} value of 74.17 μg/ml while 1,4-cineole, nerolidol, and β-caryophyllene were not nematicidal at a concentration of 5 mg/ml. The essential oil of *R. anthopogonoides* and benzyl acetone show potential for their development as possible natural nematicides for the control of the root knot nematode.

Key words: Rhododendron anthopogonoides, *Meloidogyne incognita*, Nematicidal Activity

Introduction

Rhododendron anthopogonoides Maxim. (family Ericaceae) is a shrub, growing on the damp sides of mountains and widely distributed in northwest China, especially in Sichuan, Qinghai, and Gansu provinces (Committee of Flora of China, 1999). Its flowers, leaves, and twigs are used as traditional Chinese folk medicine for treating chronic bronchitis and coronary heart disease (Jiangsu New Medical College, 1977). The crude drug contains monoterpenoids, sesquiterpenoids, triterpenoids, flavonoids, steroids, coumarins, lignans, cerebrosides, tetracyclic chromane derivatives, tannins, and alkaloids (Zhang *et al.*, 1980; Dai and Yu, 2005; Dai *et al.*, 2005; Zhao *et al.*, 2008; Iwata and Kitanaka, 2010, 2011). The essential oil of this medicinal herb has also been investigated (Lu *et al.*, 1980; Zhang *et al.*, 2003; Li *et al.*, 2004; Yang *et al.*, 2011). It was shown to inhibit the growth of bacteria (*Bacillus subtilis*, *Escherichia coli*, *Proteus vulgaris*, and *Staphylococcus aureus*) (Liu, 2007). Moreover, *R. anthopogonoides* essential oil exhibited strong insecticidal activity against maize weevils (*Sitophilus zeamais*) (Yang *et al.*, 2011). However, the available information indicates that nematicidal activity of the essential oil derived from *R. anthopogonoides* has not been the subject of any study. In this paper, we report the evaluation of the essential oil and its constituent compounds as nematicides for the control of the root knot nematode, *Meloidogyne incognita* (Kofoid and White) Chitwood.

M. incognita is the economically most important and widely distributed nematode throughout China, and a considerable crop loss is caused by this nematode. Nematode management is generally based upon chemical treatments (*e.g.* soil fumigation with methylbromide and dichloropropane), but environmental concerns and governmental regulations are now resulting in a strong interest in nematicides of natural origin (Chitwood, 2002; Rich *et al.*, 2004). One alternative is to screen compounds naturally occurring in plants, which are known as plant secondary compounds. Many plant constituents and metabolites including essential oils and monoterpenoids have been investigated for their activity

against plant-parasitic nematodes (Thoden *et al.*, 2009; Ntalli *et al.*, 2010; Echeverrigaray *et al.*, 2010; Li *et al.*, 2011; Bai *et al.*, 2011; Zhang *et al.*, 2011). A series of nematicidal substances of plant origin such as triglycerides, sesquiterpenoids, alkaloids, steroids, diterpenoids, monoterpenoids, and flavonoids have been identified (Chitwood, 2002). In part, because certain plant essential oils meet the criteria of minimum risk pesticides by the US Environmental Protection Agency (US EPA, 2004), much effort has focused on them and their constituents as potential sources of commercial nematode control products.

Material and Methods

Plant material and essential oil extraction

Fresh aerial parts (10 kg) of *R. anthopogonoides* were harvested from Guide (36.04° N and 101.43° E, Qinghai Province, China) in July 2011. A voucher specimen (CMH-Liexiangdujuan-Qinghai-2011-07) was deposited in the Department of Entomology, China Agricultural University, Beijing, China. To obtain the volatile essential oil, the air-dried samples were first ground to a powder using a grinding mill (Retsch Mühle, Haan, Germany), then soaked in water at a ratio of 1:4 (w/v) for 1 h, prior to hydrodistillation using a round-bottom flask over a period of 6 h. The volatile essential oil was collected in a specific receiver, measured, dried over anhydrous sodium sulfate, weighed, and stored in airtight containers.

Gas chromatography-mass spectrometry (GC-MS)

Gas chromatographic analysis was performed on an Agilent 6890N gas chromatograph (Agilent Technologies, Santa Clara, CA, USA) while the essential oils were identified on an Agilent Technologies 5973N mass spectrometer. It was equipped with a flame ionization detector and an HP-5MS (Agilent) capillary column (30 m x 0.25 mm x 0.25 μm). The GC settings were as follows: The initial oven temperature was held at 60 °C for 1 min and increased at 10 °C/min to 180 °C, hold for 1 min, then ramped at 20 °C/min to 280 °C, and hold for 15 min. The injector temperature was 270 °C. The samples (1 μl, diluted 1:100 in acetone) were injected, with a split ratio of 1:10. The carrier gas was helium at a flow rate of 1.0 ml/min. Spectra were scanned from *m/z* 20 to 550 at 2 scans/s. Most compounds were identified using gas chromatography by comparison of their retention indices with those given in the literature or with those of authentic compounds available in our laboratories. The retention indices were determined in relation to a homologous series of *n*-alkanes (C_8–C_{24}) under the same operating conditions. Further identification was made by comparison of their mass spectra with those stored in NIST 05 and Wiley 275 libraries or with mass spectra from the literature (Adams, 2007). Component relative percentages were calculated based on the normalization method without using correction factors.

Nematicidal toxicity bioassay

All bioassay experiments were performed under laboratory conditions at 26–28 °C. Second-stage juveniles (J2) of *M. incognita* were obtained from a pure culture that was previously initiated by egg masses and propagated on tomato (*Solanum lycopersicum*) in the glasshouse. Egg masses were hand-picked using sterilized forceps from heavily infected roots (40 d after incubation), washed in distilled water, and placed in 15-mesh sieves (8 cm in diameter) containing crossed layers of tissue papers in Petri dishes with water level in contact with the egg masses. The set-up was subsequently incubated at 25–26 °C to hatch J2. Only juveniles collected within 48 h were used. Range-finding studies were run to determine the appropriate testing concentrations. A serial dilution of the essential oil of *R. anthopogonoides* (six concentrations, dissolved in 10 μl ethanol) and pure compounds (six concentrations) was prepared in H_2O with 2% dimethyl sulfoxide (DMSO). Aliquots of H_2O (20 μl) containing ca. 100 J2 were transferred to vials to which 980 μl of the solution containing ethanol extract or pure compounds were added. The vials were kept on a hood at 25 °C. The inactive nematodes were counted every 24 h for 72 h. After the last count, the inactive juveniles were maintained in distilled H_2O for 24 h to observe their revival. Six repetitions for each treatment were performed using H_2O and a solution of 2% (v/v) DMSO in H_2O as well as 2% DMSO in H_2O containing 10 μl/ml ethanol as control. The experiments were repeated three times. Results from all replicates for the pure compounds and ethanol extract were subjected to probit analysis using the PriProbit Program V1.6.3 to determine LC_{50} values (Sakuma, 1998). Carbofuran was purchased from the Na-

tional Center of Pesticide Standards (Shenyang, China) and used as a positive control.

Chromatography

The crude essential oil (20 ml) was chromatographed on a silica gel (Merck 9,385, 1,000 g; Merck Chemicals Co., Ltd., Shanghai, China) column (85 mm i.d., 850 mm in length) by gradient elution with a mixture of solvents (*n*-hexane, *n*-hexane/ethyl acetate, and ethyl acetate). Fractions of 500 ml each were collected and concentrated at 40 °C, and similar fractions according to thin-layer chromatography (TLC) profiles were combined to yield 15 fractions. Fractions 4, 7, 9, and 11, with similar TLC profiles, were pooled and further purified by preparative TLC (PTLC) to obtain four pure compounds determined by their structure as 1,4-cineole (**1**) (44 mg), benzyl acetone (4-phenyl-2-butanone, **2**) (71 mg), *β*-caryophyllene (**3**) (55 mg), and nerolidol (**4**) (39 mg). The structures of the compounds were elucidated based on high-resolution electron impact mass spectrometry (HR-EI-MS) and nuclear magnetic resonance (NMR).

1,4-Cineole (**1**): Colourless oil. – ^{1}H NMR (500 MHz, $CDCl_3$): δ = 0.93 (3H, s, 9-CH_3), 0.94 (3H, s, 10-CH_3), 1.42 (3H, s, 7-CH_3), 1.48 – 1.67 (8H, m, 2-CH_2, 3-CH_2, 5-CH_2, 6-CH_2), 2.04 (1H, m, J = 13.7, 6.8 Hz, H-8). – ^{13}C NMR (125 MHz, $CDCl_3$): δ = 18.12 (C-9), 18.18 (C-10), 21.22 (C-7), 32.92 (C-3, C-5), 33.06 (C-2, C-6), 37.22 (C-8), 82.96 (C-1), 89.68 (C-4). – EI-MS: *m/z* (%) = 154 (26), 125 (29), 111 (73), 71 (60), 69 (35), 55 (41), 43 (100), 41 (44), 27 (22); $C_{10}H_{18}O$. – The data matched with those of previous reports (Asakawa *et al.*, 1988; Yang *et al.*, 2011).

Benzyl acetone (**2**): Colourless oil. – ^{1}H NMR (500 MHz, $CDCl_3$): δ = 2.11 (3H, s, 9-CH_3), 2.73 (2H, t, J = 8.0 Hz, 7-CH_2), 2.90 (2H, t, J = 8.0 Hz, 8-CH_2), 7.19 (1H, dd, J = 8.0 Hz, 4-H), 7.21 (2H, d, J = 12.0, 8.0 Hz, H-2, H-6), 7.30 (2H, dd, J = 12.0, 8.0 Hz, H-3, H-5). – ^{13}C NMR (125 MHz, $CDCl_3$): δ = 29.74 (C-10), 29.95 (C-7), 45.02 (C-8), 126.12 (C-4), 128.35 (C-3, C-5), 128.51 (C-2, C-6), 141.10 (C-1), 207.62 (C-9). – EI-MS: *m/z* (%) = 149 (8), 148 (73), 133 (14), 105 (81), 104 (11), 91 (60), 79 (13), 78 (11), 77 (17), 43 (100), 51 (11); $C_{10}H_{12}O$. – The ^{1}H and ^{13}C NMR data were in agreement with the reported data (Black *et al.*, 2006; Fox *et al.*, 2006).

β-Caryophyllene (**3**): Colourless oil. – ^{1}H NMR (500 MHz, $CDCl_3$): δ = 0.97 (3H, s, H-13), 1.00 (3H, s, H-14), 1.46 (1H, m, H-2a), 1.51 (1H, m, H-3a), 1.61 (3H, s, H-15), 1.63–1.66 (2H, m, H-10), 1.68 (1H, m, H-3b), 1.70 (1H, m, H-2b), 1.91 (1H, td, J = 12.3, 4.6 Hz, H-6a), 2.00 (1H, brd, J = 9.1 Hz, H-6b), 2.08 (1H, brd, J = 11.6 Hz, H-1), 2.20 (1H, t, J = 6.0 Hz, H-7a), 2.33 (1H, m, H-7b), 2.35 (1H, m, J = 9.3 Hz, H-9), 4.82 and 4.94 (2H, s, H-12a and H-12b), 5.29 (1H, m, H-5). – ^{13}C NMR (125 MHz, $CDCl_3$): δ = 16.3 (C-15), 22.6 (C-14), 28.4 (C-2), 29.4 (C-6), 30.1 (C-13), 33.0 (C-11), 34.8 (C-7), 40.0 (C-3), 40.4 (C-10), 48.5 (C-9), 53.6 (C-1), 111.6 (C-12), 124.3 (C-5), 135.5 (C-4), 154.7 (C-8). – EI-MS: *m/z* (%) = 204 $[M]^+$ (4), 189 (10), 161 (18), 147 (15), 133 (50), 120 (24), 105 (39), 93 (65), 91 (66), 79 (60), 69 (63), 55 (35), 41 (100); $C_{15}H_{24}$. – The ^{1}H and ^{13}C NMR data were in agreement with the reported data (Kitajima *et al.*, 1989).

Nerolidol (**4**): Colourless oil. – ^{1}H NMR (500 MHz, $CDCl_3$): δ = 1.29 (3H, s, 15-CH_3), 1.56 (2H, m, J = 9.8, 6.1 Hz, 9-CH_2), 1.59 (3H, s, 1-CH_3), 1.60 (3H, s, 13-CH_3), 1.69 (3H, s, 14-CH_3), 1.76 (1H, br, 10-OH), 1.97 – 2.09 (6H, m, 4-CH_2, 5-CH_2, 8-CH_2), 5.04–5.12 (2H, m, 12-CH_2), 5.15 (1H, t, 3-H), 5.22 (1H, dd, J = 17.3, 1.2 Hz, 7-H), 5.92 (1H, m, J = 17.3, 10.7 Hz, 11-H). – ^{13}C NMR (125 MHz, $CDCl_3$): δ = 16.01 (C-14), 17.69 (C-8), 22.72 (C-1), 25.71 (C-4), 26.63 (C-13), 27.86 (C-15), 39.70 (C-5), 42.04 (C-9), 73.49 (C-10), 111.67 (C-12), 124.22 (C-3), 124.24 (C-7), 131.41 (C-2), 135.52 (C-6), 145.05 (C-11). – EI-MS: *m/z* (%) = 204 (3), 161 (11), 136 (15), 107 (24), 93 (50), 81 (27), 71 (37), 69 (100), 55 (26), 43 (28), 41 (65); $C_{15}H_{26}O$. – The ^{1}H and ^{13}C NMR data matched with those of previous reports (Cuca Suarez *et al.*, 2002; Yang *et al.*, 2011).

Results and Discussion

Essential oil analysis

The yield of the yellow essential oil of *R. anthopogonoides* was 0.92% (v/w), with a density of 0.90 g/ml. A total of 42 compoounds were identified in the essential oil of *R. anthopogonoides* leaves and stems, accounting for 95.48% of the total oil (Table I). The main compounds (Fig. 1) of the essential oil were benzyl acetone (34.41%), nerolidol (10.19%), 1,4-cineole (8.41%), *β*-caryophyllene (5.63%), *γ*-elemene (5.10%), and spathulenol (3.06%) (Table I). Monoterpenoids

Table I. Chemical composition of the essential oil of *Rhododendron anthopogonoides*.

No.	Compound	RI[a]	Content (%)
1	*α*-Pinene	939	1.38
2	Camphene	954	0.31
3	*β*-Pinene	974	0.22
4	Morillol	980	0.78
5	*β*-Myrcene	991	0.24
6	1,4-Cineole	1018	8.41
7	ρ-Cymene	1025	0.67
8	(+)-Limonene	1029	1.42
9	1,8-Cineole	1031	0.28
10	(*E*)-*β*-Ocimene	1068	0.29
11	Linalool	1097	0.31
12	Camphor	1143	0.70
13	Citronellal	1158	0.11
14	4-Terpineol	1177	1.21
15	*α*-Terpineol	1188	0.54
16	Benzyl acetone	1218	34.41
17	Citronellol	1228	1.15
18	Linalool acetate	1253	1.89
19	*γ*-Pyronene	1338	0.02
20	Longipinene	1350	0.11
21	Eugenol	1356	0.88
22	*α*-Ylangene	1370	0.42
23	*α*-Copaene	1374	0.59
24	*β*-Cubebene	1387	1.79
25	Benzyl isovalerate	1395	0.91
26	*α*-Gurjunene	1411	0.81
27	*β*-Caryophyllene	1420	5.63
28	*β*-Ylangene	1423	0.42
29	*β*-Gurjunene	1434	1.97
30	*γ*-Elemene	1437	5.10
31	*2*,3-Dimethylnaphthalene	1443	0.29
32	*β*-Farnesene	1453	0.23
33	1,5,9,9-Tetramethyl-, *Z*,*Z*,*Z*-1,4,7-cycloundecatriene	1456	0.21
34	*β*-Chamigrene	1478	0.67
35	*α*-Curcumene	1483	0.21
36	*β*-Guaiene	1487	2.31
37	*α*-Farnesene	1505	1.43
38	*β*-Sesquiphellandrene	1523	1.24
39	Nerolidol	1567	10.19
40	Germacrene D-4-ol	1574	2.13
41	Spathulenol	1578	3.06
42	*γ*-Eudesmol	1621	1.21
	Total		95.48
	Monoterpenoids		19.13
	Sesquiterpenoids		39.37
	Others		36.98

[a] RI, retention index as determined on an HP-5MS column using a homologous series of *n*-hydrocarbons as reference.

represented 17 of the 42 compounds, corresponding to 19.13% of the whole oil while 20 of the 42 constituents were sesquiterpenoids (39.37% of the crude essential oil).

Nematicidal activity

The essential oil of *R. anthopogonoides* possessed nematicidal activity against the root knot nematode (*M. incognita*) with an LC_{50} value of 130.11 μg/ml. Compared with the synthetic insecticide carbofuran (also used as a nematicide; LC_{50} 72.29 μg/ml), the essential oil exhibited half the level of toxicity against *M. incognita* (Table II). Of the four isolated main compounds of the essential oil of *R. anthopogonoides*, benzyl acetone exhibited strong nematicidal activity against *M. incognita* with an LC_{50} value of 74.17 μg/ml while 1,4-cineole, *β*-caryophyllene, and nerolidol did not exhibit nematicidal activity against *M. incognita* at a concentration of 5 mg/ml (Table II). Compared with the positive control, carbofuran, benzyl acetone showed the same level of nematicidal activity against *M. incognita* and exhibited two times stronger activity than the crude essential oil of *R. anthopogonoides*. It is suggested that the nematicidal activity of the essential oil of *R. anthopogonoides* may be attributed to its main constituent benzyl acetone. Benzyl acetone is a liquid with a sweet, flowery smell (similar to the smell of jasmine and strawberry) that is

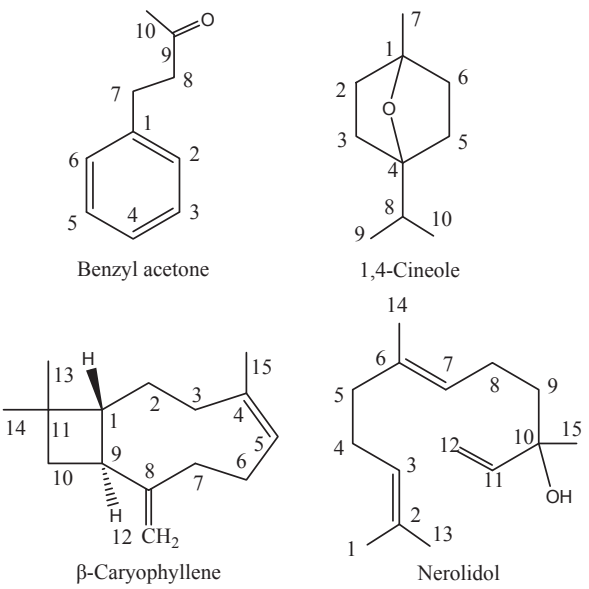

Fig. 1. Chemical structures of the compounds isolated from *Rhododendron anthopogonoides*.

Table II. Nematicidal activity of the essential oil of *Rhododendron anthopogonoides* aerial parts and its main compounds against *Meloidogyne incognita*.

Treatment	Concentration [μg/ml]	N[a]	LC_{50} [μg/ml]	95% FL[b]	Slope ± SE	Chi-square test
Essential oil	12.5 – 200.0	3478	130.11	59.08 – 214.34	0.62 ± 0.05	7.58[c]
Benzyl acetone	6.5 – 100.0	3347	74.17	56.86 – 112.93	0.56 ± 0.04	2.95[c]
β-Caryophyllene	–		>5,000.00	–	–	–
1,4-Cineole	–		>5,000.00	–	–	–
Nerolidol	–		>5,000.00	–	–	–
Carbofuran	5.0 – 100.0	3423	72.29	37.86 – 117.97	0.34 ± 0.03	13.57[c]

[a] *N*, number of second-stage juveniles of *M. incognita* used in the bioassay.
[b] FL, fiducial limits.
[c] $p < 0.05$.

considered to be the most abundant attractant compound in some flowers, *e.g. Nicotiana attenuata* (Baldwin *et al.*, 1997). However, benzyl acetone was found to exhibit repellent activity against honeybees (*Apis florea*) (Gupta, 1987) and to possess strong insecticidal activity against maize weevils (*Sitophilus zeamais*) (Yang *et al.*, 2011). This compound has been found here for the first time to exhibit nematicidal activity against *M. incognita*. Benzyl acetone seems to have low toxicity against mammals [*e.g.* in the mouse, the oral LD_{50} is 1,590 mg/kg and the intraperitoneal LD_{50} is 583 mg/kg (Li *et al.*, 1980)]. *R. anthopogonoides* aerial parts are commonly used as traditional Chinese folk medicine for treating chronic bronchitis and coronary heart disease (Jiangsu New Medical College, 1977) and its active component benzyl acetone was found to be effective as an antitussive in Chinese traditional medicine (Li *et al.*, 1980). However, no information on safety data of the essential oil and the compound after human consumption was available.

The above findings suggest that the essential oil of *R. anthopogonoides* can play an important role in plant protection and also reduce the risks associated with synthetic nematicides. However, for the practical application of the essential oil or benzyl acetone as a novel nematicide, further studies on the safety of these agents for humans and on the development of formulations are necessary to improve their efficacy and stability and to reduce their cost.

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