Screening and isolation of a nematicidal sesquiterpene from *Magnolia grandiflora* L.

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Abstract: The ethanolic extracts from 30 plant species were tested for their nematicidal activity against nematodes *Bursaphelenchus xylophilus* (Steiner & Buhrer) Nickle and *Panagrellus redivivus* (L.) Goodey. The leaf extract of *Magnolia grandiflora* L. exhibited the strongest nematicidal activity against both nematodes, causing 73 and 100% mortality respectively within 48 h at 5 mg mL^{-1}. A new nematicidal sesquiterpene was obtained from the leaves of *M. grandiflora*. The compound was determined to be 4,5-epoxy-1(10)*E*,11(13)-germacradien-12,6-olide, based on spectroscopic methods including 2D NMR techniques. The median lethal concentrations (LC_{50}) of the compound against *B. xylophilus* and *P. redivivus* were 71 and 46 mg L^{-1} respectively at 48 h. This is the first report of Magnoliaceae species with nematicidal activity.

Keywords: nematode; plant extracts; sesquiterpene; *Magnolia grandiflora* L

1 INTRODUCTION

Plant parasite nematodes have inflicted serious damage on agricultural crops and plants. The pinewood nematode, *Bursaphelenchus xylophilus* (Steiner & Buhrer) Nickle causes disastrous diseases in the pinewood, resulting in $1 billion economic losses per year.[1] In the past, mainly synthetic compounds have been used for plant protection, but the side effects of many of these pesticides, such as resistance, residues in plants and contamination of groundwater,[2] have led to a drastic reduction in efficient commercial nematicides. Plants, as one of main biological resources, are always a large source of new agricultural chemicals. The potential for nematicidal activity of plants and their products as an alternative to traditional nematicides has been studied by many researchers.[3–7] The nematicidal principles of plant origin in the form of substances such as triglycerides, sesquiterpenes, alkaloids, steroids, essential oils, diterpenes and flavonoids have been identified.[8–13] In the present work, leaf and branch extracts of 30 plant species were tested for their nematicidal activity, and a new sesquiterpene with nematicidal activity was isolated from *Magnolia grandiflora* L.

2 MATERIALS AND METHODS

2.1 Plant materials and plant extracts

A total of 48 materials including leaf and branch tissue of 30 species (Table 1) were collected from Kunming Institute of Botany, PR China.

The plant materials were washed with running tap water and then dried in an oven at 50 °C. Dry plant materials (10 g) were chopped into small pieces and extracted 3 times with 80% ethanol (100 mL) at room temperature (72 h each time). The ethanolic extracts were filtered and concentrated under vacuum, and the residues were then dissolved in 80% ethanol. These samples were conserved at 4 °C prior to use.

2.2 Spectroscopic measurements and chromatography

NMR experiments were carried out on a Bruker DRX-500 spectrometer. Mass spectra were recorded on a VG Auto-Spec-3000 mass spectrometer. Melting points were measured without correction on a Kofler microscope (Reichert). Infrared (IR) spectra were measured on a Paragon 1000pc spectrometer. Optical rotations were measured on a Jasco DIP-370 digital polarimeter. Column chromatography was carried out on silica gel (200–300 mesh; Qingdao Marine Chemical Factory, Qingdao, PR China.) and Sephadex LH-20 (Pharmacia). Thin-layer chromatography (TLC) was performed on silica gel (Si gel G; Qingdao Marine Chemical Factory, Qingdao, PR China.), and the spots were visualized under 5% vitriol ethanol solution.

2.3 Isolation and structural characterization of sesquiterpene from *Magnolia grandiflora* leaves

The dried leaves (40 g) of *M. grandiflora* were extracted by the same method as described in Section 2.1 to produce a crude residue (2.9 g). This was dissolved in water and extracted using ethyl acetate. Bioassay results showed that the ethyl acetate fraction was the

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Table 1. Plant species assessed

Plant species	Family	Plant part
Argemone mexicana L	Papaveraceae	Branch, leaf
Melia azedarach L	Meliaceae	Branch, leaf
Michelia hedyosperma Law	Magnoliaceae	Branch, leaf
Magnolia grandiflora L	Magnoliaceae	Branch, leaf
Fraxinus malacophylla Hemsl	Oleaceae	Branch, leaf
Maesa indica Wall	Myrsinaceae	Branch, leaf
Chaenomeles lagenaria Koidz	Rosaceae	Branch, leaf
Cotoneaster horizontalis Decne	Rosaceae	Branch
Crataegus pinnatifida Bge	Rosaceae	Branch, leaf
Nerium indicum Mill	Apocynaceae	Branch, leaf
Pinus yunnanensis Franch	Pinaceae	Branch, leaf
Coriaria sinica Maxim	Coriariaceae	Branch, leaf
Camptotheca acuminata Decne	Nyssaceae	Branch
Asarum chingchengense C.Y.Cheng & C.S.Yang	Aristolochiaceae	Leaf
Edgeworthia chrysantha Lindl	Thymelaeaceae	Branch
Glochidion puberum (L.) Hutch	Euphorbiaceae	Branch
Eucommia ulmoides Oliver	Eucommiaceae	Branch, leaf
Aleurites fordii Hemsl	Euphorbiaceae	Branch
Sapium sebiferum (L.) Roxb	Euphorbiaceae	Branch, leaf
Podocarpus macrophyllus (Thunb.) D.Don	Podocarpaceae	Branch
Elaeagnus viridis Serv. var. delavayi	Elaeagnaceaw	Branch, leaf
Zingiber striolatum Diels	Zingiberaceae	Branch, leaf
Peperomia tetraphylla (G. Forest) Hook. & Arn	Piperaceae	Branch, leaf
Hypericum monogynum L.	Guttiferae	Branch
Adiantum capillus-veneris L.	Adiantaceae	Leaf
Pachysandra axillaries Franch	Buxaceae	Branch
Buxus microphylla Sieb. & Zucc. ssp. sinica (Rehd. & Wils.) Hatusima	Buxaceae	Branch, leaf
Lagerstroemia indica L.	Lythraceae	Branch, leaf
Lespedeza formosa (Vog.) Koehne	Leguminosae	Leaf
Punica granatum L.	Punicaceae	Branch

active fraction. In a further study, bioassay guide was used in the isolation process. The ethyl acetate fraction (0.89 g) was chromatographed on a silica gel column (20 g, 200–300 mesh) eluting with petroleum ether + acetone (9 + 1, 8 + 2, 7 + 3 and 2 + 1 by volume) to yield fractions A_1 to A_8. Active fraction A_4 was purified on a Sephadex LH-20 column eluted with acetone to give fractions A_{41} to A_{46}. Active fraction A_{44} was further chromatographed on a silica gel column (8 mg, 200–300 mesh) eluting with petroleum ether + acetone (9 + 1 by volume) to obtain fraction A_{443} which was repeatedly purified on a Sephadex LH-20 column eluted with acetone to afford 16 mg of active compound. The compound was a colourless powder; $[\alpha]_D^{20} = -6.25$ (*c* 0.8, acetone); m.p. 260 °C; NMR spectral data are given in Table 2; ESI-MS: 249 ($[M + H]^+$). IR (KBr): 3444, 2936, 1772, 1668, 1256, 1152, 1080 cm^{-1}.

2.4 Culture of nematodes

The culture methods for the saprophytic nematode *Panagrellus redivivus* (L.) Goodey and the pinewood nematode *B. xylophilus* were as described previously.[14]

2.5 Assay of nematicidal activity

2.5.1 Nematicidal activity of plant extracts

Each extract was dissolved at 5 mg mL^{-1} in sterile water. A sample (2 mL) was added to a 6 cm diameter petri dish containing 100–150 nematodes. Each treatment was replicated 3 times, with the same concentration of ethanol used as control. Dead and active nematodes were counted after 24 and 48 h. The nematodes were considered to be dead when they did not move on physical stimuli with a fine needle.[15,16] The mean percentage mortality was calculated.

2.5.2 Nematicidal activity of the compound

Five percent acetone water solutions of the compound at 200, 100, 50 and 25 mg L^{-1} were assayed for nematicidal activity by the method described in Section 2.5.1. Avermectin was used as standard. The experiments to assay nematicidal activity were repeated twice.

2.6 Statistical analysis

To compare the nematicidal activities of the compound against two different nematodes, *P. redivivus* and *B. xylophilus*, and between different exposure times of 24 and 48 h, data were subjected to independent sample t-testing using ANALYZE (SPSS/version 11.0 software; SPSS, Chicago, IL, USA). Data on proof mortality M were changed to $\sin^{1/2}(M)$ before analysis.

To describe the nematicidal effects of the compound against *P. redivivus* and *B. xylophilus*, LC_{50} values were calculated according to probit analysis.[17] Regression analysis were also conducted by SPSS for a linear model. Data on proof mortality of nematodes were transformed into probit values, and the concentrations C of the compound were changed to $\log_{10}(C)$ before analysis.

3 RESULTS AND DISCUSSION

3.1 Isolation and structure determination of sesquiterpene from *Magnolia grandiflora*

The sesquiterpene was obtained as colourless powder. $[\alpha]_D^{20} = -6.25$ (*c* 0.8, acetone). The molecular formula was established to be $C_{15}H_{20}O_3$ by analysis of its ESI-MS (*m/z* 249 $[M + H]^+$) and DEPT spectra (Table 2). ^{13}C NMR and DEPT spectra (Table 2) indicated that the compound contained a sesquiterpene lactone skeleton[16] leading to a total of 15 carbon signals – two CH_3, five CH_2, four CH and four quaternary carbons, which contained a carboxyl group (δ 170) and two carbon–carbon double bonds (δ 126.6 and 135.5, 122.0 and 140.2). In the HMBC experiment (Table 2), the protons of methylene at δ 6.36 (H_α-13) and 5.66 (H_β-13) displayed correlation with the carbons at δ 48.6 (C-7), 140.2 (C-11) and 170.1

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Table 2. NMR data of 4,5-epoxy-1(10)*E*,11(13)-germacradien-12,6-olide (in $CDCl_3$)

Position	^1H	^{13}C	HMBC	COSY
1	5.26 (br, d, $J = 11$)	126.2	42.1, 17.8	2.48
2	2.48 (dt, $J = 13.5, 5.5$)	25.0	37.3, 126.2, 135.5	5.26
	2.22, m		18.2, 62.3,	–
3	2.22, m	37.3	18.2, 62.3,	–
	1.31, m		18.2, 25.0, 62.3, 67.3	–
4	–	62.3	–	–
5	2.83 (d, $J = 6.1$)	67.3	140.2(w), 83.3, 62.3,	–
6	3.91 (t, $J = 8.5$)	83.3	62.3, 67.3, 48.6(w), 31.6	2.83
7	2.83 (d, $J = 6.1$)	48.6	140.2, 83.3	3.91
8	2.22, m	31.6	42.1, 48.6, 126.2, 135.5, 140.2	–
	1.80, m		126.2, 135.5	–
9	2.42, m	42.1	17.8, 31.6,	–
	2.24, m		17.8, 25.0, 31.6, 126.2, 135.5	–
10	–	135.5	–	–
11	–	140.2	–	–
12	–	170.1	–	–
13	6.36 (d, $J = 3.3$)	122.0	48.6, 140.2, 170.1	–
	5.66 (d, $J = 3.2$)		48.6, 170.1	–
14	1.78, s	17.8	31.6, 42.1, 126.2, 135.5	–
15	1.34, s	18.2	37.3, 62.3, 67.3	–

(C-12), the proton of methine at δ 2.83 (H-7) displayed correlation with the carbons at 140.2 (C-11) and 83.3 (C-6) and the proton of methine at δ 3.91 (H-6) displayed correlation with the carbons at 67.3 (C-5) and 48.6 (C-7), which provided a fragment unit of a furan lactone ring. Methyl protons at δ 1.78 (H-14) signal revealed couplings to carbons at δ 31.6 (C-8), 42.1 (C-9), 126.2 (C-1) and 135.5 (C-10), the protons of methylene at δ 2.22 (H_α-8) and 1.80 (H_β-8) correlated with the carbons at δ 17.8 (C-14), 42.1(C-9), 48.6 (C-7), 83.3(C-6), 126.2 (C-1), 135.5 (C-10) and 140.2 (C-11), the proton of methine at δ 5.26 (H-1) correlated with the carbons at δ 42.1 (C-9) and 17.8 (C-14) and the protons of methylene at δ 2.42 (H_α-9) and 2.24 (H_β-9) correlated with the carbons at δ 17.8 (C-14), 25.0 (C-2), 31.6 (C-8), 126.2 (C-1) and 135.5 (C-10). The oxygenated methine proton at δ H 2.83 (C-5) correlated with the carbons at δ 83.3 (C-6) and 62.3 (C-4), and the methyl protons at δ 1.34 (H-15) revealed coupling to carbons at δ 37.3 (C-3), 62.3 (C-4) and 67.3 (C-5). The NOESY experiment showed NOE correlations between H-6 and H-15, and between H-15 and H-5, H-7, which provided its relative stereochemistry, and the configuration of the double bond between C-1 and C-10 should be an (*E*) C=C double bond according to the coupling constant.[18,19] Therefore, the compound was determined to be 4,5-epoxy-1(10)*E*,11(13)-germacradien-12,6-olide. The skeleton of the compound was the same as that of parthenolide,[19] but the optical activities and melting point were considerably different, which indicated that the configurations also were different (Fig. 1).

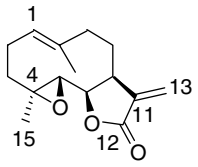

Figure 1. Structure of the sesquiterpene.

3.2 Nematicidal activity of plant extracts

The results presented in Table 3 indicated that nematicidal activity varied among plant species and plant parts. Among them, leaf extracts of *Magnolia grandiflora* and *Michelia hedyosperma* and branch extract of *Nerium indicum* showed nematicidal activity against both *P. redivivus* and *B. xylophilus* after 48 h, which accounted for 6.2% of all crude extracts. It was also observed that the leaf extract of *Magnolia grandiflora* was the most effective, followed by leaf extract of *Michelia hedyosperma* and branch extract of *Nerium indicum*. Four plant extracts showed nematicidal activity against *P. redivivus*, but none had an obvious effect against *B. xylophilus*. Those extracts that had little or no nematicidal activity are not shown in Table 3.

Results obtained from the present study further support the view that some plants are promising sources

Table 3. Effect of plant extracts (5 mg L^{-1} in water) on the mortality (%) of the nematodes

		P. redivivus		*B. xylophilus*	
Plant species	Plant part	24 h	48 h	24 h	48 h
Sapium sebiserum	Branch	43	62	0	0
Magnolia grandiflora L.	Leaf	87	100	51	73
Michelia hedyosperma	Leaf	84	100	44	66
Nerium indicum	Branch	86	100	22	45
Zingiber striolatum	Branch	62	82	0	0
Punica granatum	Branch	61	75	0	0
Edgeworthia chrysantha	Branch	40	57	0	0
Control (4% ethanol)	–	1.2	2	0	0

of bionematicides. Nematicidal activity differs significantly among the 30 plants selected. The extracts of *Michelia hedyosperma* and *Magnolia grandiflora* showed the strongest nematicidal activities against the tested nematodes. Furthermore, the two plants belong to the same family, Magnoliaceae. This is the first report of Magnoliaceae species with nematicidal activity. It is suggested that more plants belonging to this family should be screened to search for new sources of nematicidal substances. Results also indicate that the nematicidal effect varies among different parts of the plant, which may suggest that the different parts comprise different chemical components.

3.3 Nematicidal activity of 4,5-epoxy-1(10)*E*,11(13)-germacradien-12,6-olide

The compound caused significant mortality to *P. redivivus* and *B. xylophilus* at 200, 100 and 50 mg L^{-1} (Table 4). Nematicidal effects varied with concentration and exposure time. The nematicidal activity differed significantly between exposure times of 24 and 48 h at the same concentration (Table 5). The mortality of *P. redivivus* was significant higher than that of *B. xylophilus* at the same concentration with the same exposure time (Table 6). Probit value of proof mortality showed a linear increase with increasing $\log_{10}(C)$. The LC_{50} values of the compound against *P. redivivus* and *B. xylophilus* were 46 and 71 mg L^{-1} respectively at 48 h; the 95% fiducial limits of these LC_{50} values were (41, 52) and (68, 89). Avermectin showed no effect against *P. redivivus* at 200 mg L^{-1} but caused high mortality on *B. xylophilus*, with an LC_{50} value of 0.2 mg L^{-1} at 48 h; the 95% fiducial limits of this LC_{50} value ranged from 0.18 to 0.24 mg L^{-1}.

Sesquiterpenes from plants or microbes have been shown to have activity against nematodes.[20–22] Results from the present experiment demonstrate that the sesquiterpene from leaves of *Magnolia grandiflora* caused high mortality on *P. redivivus* and *B. xylophilus*. It is worth mentioning that the chemical constituents of *Magnolia grandiflora* have been studied for a long time,[23–26] but a new sesquiterpene was obtained in the present study on nematicidal activity, suggesting that it is necessary to utilize plant resources in searching for new active compounds by choosing a suitable biological screening model.

Table 4. Effect of 4,5-epoxy-1(10)*E*,11(13)-germacradien-12,6-olide on the mortality (%) of the two nematodes

	P. redivivus		*B. xylophilus*	
Concentration (mg L^{-1})	24 h	48 h	24 h	48 h
200	92	100	72	83
100	77	87	48	61
50	34	53	22	36
25	16	24	8.5	13
Control (5% acetone)	1.5	2.0	0	0

Table 5. Variation in percentage mortality of nematodes between 24 and 48 h at each concentration of 4,5-epoxy-1(10)*E*,11(13)-germacradien-12,6-olide

	200 mg L^{-1}	100 mg L^{-1}	50 mg L^{-1}	25 mg L^{-1}
P. redivivus	−13.5[a]	−7.9[b]	−12.9[a]	−2.9[c]
B. xylophilus	−8.9[b]	−7.5[b]	−11.2[a]	−4.6[c]

[a] $P < 0.001$.
[b] $P < 0.01$.
[c] $P < 0.05$.

Table 6. Difference in mortalities of *Panagrellus redivivus* and *Bursaphelenchus xylophilus* exposed to various concentrations of 4,5-epoxy-1(10)*E*,11(13)-germacradien-12,6-olide for the same times

	200 mg L^{-1}	100 mg L^{-1}	50 mg L^{-1}	25 mg L^{-1}
24 h	11.0[a]	16.3[a]	8.6[b]	4.5[c]
48 h	47.7[a]	19.7[a]	12.7[a]	6.8[b]

[a] $P < 0.001$.
[b] $P < 0.01$.
[c] $P < 0.05$.

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