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PII: S0378-8741(18)31371-0
DOI: <https://doi.org/10.1016/j.jep.2018.06.026>
Reference: JEP11415

To appear in: *Journal of Ethnopharmacology*

Received date: 1 May 2018
Revised date: 13 June 2018
Accepted date: 18 June 2018

Cite this article as: Maoxuan Liu, Cedrick Veryser, Jing-Guang Lu, Tom Wenseleers, Wim M. De Borggraeve, Zhi-Hong Jiang and Walter Luyten, Bioassay-guided isolation of active substances from *Semen Torreya* identifies two new anthelmintic compounds with novel mechanism of action, *Journal of Ethnopharmacology*, <https://doi.org/10.1016/j.jep.2018.06.026>

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Bioassay-guided isolation of active substances from *Semen Torreyae* identifies two new anthelmintic compounds with novel mechanism of action

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Abstract:

Ethnopharmacological relevance:

Semen Torreyae, the seeds of *Torreya grandis* Fortune ex Lindley (Cephalotaxaceae) is a well-known traditional Chinese medicinal plant recorded in the Chinese Pharmacopeia (2010 version). It is widely used for treating intestinal parasites in China, owing to its desirable efficacy and safety. However, the anthelmintic compounds in *Semen Torreyae* have not yet been identified.

Aim of the study:

This study aims to identify the compounds active against helminths from *Semen Torreyae*. In addition, we tested whether *C. elegans* strains resistant to currently-used

anthelmintic drugs showed cross-resistance to these compounds.

Methods:

A bioassay-guided isolation of anthelmintic compounds from *Semen Torreya* was performed using a *Caenorhabditis elegans* (*C. elegans*) testing model. The structures of active compounds were elucidated by a combination of GC-MS, high resolution MS, and NMR. The median-effect method was employed to generate a combination index (CI) to evaluate the synergistic effect of the anthelmintic compounds. A panel of *C. elegans* mutant strains resistant against the major anthelmintic drug classes was used to study the cross-resistance to currently-used anthelmintic drugs. A panel of transient receptor potential (TRP) channel mutant strains was also tested to explore the possible mechanisms of action of the anthelmintic compounds.

Results

The bioassay-guided isolation led to two active compounds, *i.e.* galangal acetate (IC₅₀: 58.5 ± 8.9 µM) and miogadial (IC₅₀: 25.1 ± 5.4 µM). The combination of galangal acetate and miogadial resulted in a synergistic effect at IC₅₀, IC₇₀, and IC₉₀ levels (CIs < 1). Galangal acetate and miogadial demonstrated similar activity against drug-resistant *C. elegans* strains compared to the wild-type strain. In addition, none of the TRP mutants was significantly resistant to galangal acetate or miogadial compared to wild type worms.

Conclusions:

We identified the bioactive compounds from *Semen Torreya* responsible for its anthelmintic activity: galangal acetate and miogadial. The two anthelmintic compounds demonstrated a synergistic effect against *C. elegans*. Galangal acetate and miogadial are unlikely to act on the targets of currently-used anthelmintics (ivermectin, levamisole, benomyl and aldicarb), and an action on TRP channels appears to be ruled out as well. In summary, galangal acetate and miogadial are promising anthelmintic hits worth further investigation.

Graphical abstract

Keywords: anthelmintic activity, *Caenorhabditis elegans*, *Semen Torreyae*, galangal acetate, miogadial, synergy

1. Introduction

Parasitic nematodes infect about 2 billion people worldwide, especially in developing regions. The infections can have serious effects on human growth, nutrition, cognition, school performance, work productivity, and pregnancy. Helminth infections also indirectly cause a considerable disease burden via impairment of the immune system, leading to increased severity/infectivity of malaria, HIV/AIDS, and tuberculosis (Brindley and Loukas, 2017; Hu et al., 2010; Pina-Vazquez et al., 2017; Weaver et al., 2017). In addition, parasitic nematodes can cause dramatic economic losses in livestock and agriculture (Egualé et al., 2011; Kozan et al., 2016).

In the absence of vaccines for intestinal nematodes, the treatment of infections conventionally relies on chemotherapy. However, the development of resistance has diminished the success of conventional anthelmintic drugs for the control of helminths in livestock, and occasionally in human (Egualé et al., 2011; Geurden et al., 2015; Laing et al., 2016; Zhu et al., 2013). It may only be a matter of time before resistance becomes common in parasites of humans (Alum et al., 2010; Taman and Azab, 2014). Furthermore, side effects due to inappropriate dosing, and risk of drug residues in animal products, have prompted usage restrictions and de-registrations for several nematicides (Bachaya et al., 2009; Burns et al., 2017). Therefore, there is an urgent need to discover new anthelmintics with a novel mechanism of action for parasitic nematodes (Vercruysse et al., 2018).

Medicinal plants have been used traditionally to treat intestinal parasites for millennia

owing to their efficacy and safety. They can be a rich source of anthelmintic drug candidates (Agyare et al., 2014; Bajin Ba Ndob et al., 2016; Kozan et al., 2016; Kumarasingha et al., 2014; Romero-Benavides et al., 2017). However, the anthelmintic compounds in most of these medicinal plants have not yet been identified. One such plant is *Semen Torreyae*, the seeds of *Torreya grandis* Fortune ex Lindley (Cephalotaxaceae). *Semen Torreyae* is a well-known traditional Chinese medicinal plant recorded in the Chinese Pharmacopeia (2010 version), and is used to treat parasitic diseases (Saeed et al., 2010; Zhou, 2014). Its safety and efficacy for treating intestinal parasites has been extensively confirmed on humans by clinical studies (Cao, 1959; Chen et al., 2014; Mei, 1957; Xiao, 1985; Yu et al., 1957). Moreover, *Semen Torreyae* has also been identified to be effective against *Nippostrongylus brasiliensis* *in vitro* (Chen and Zhen, 2000). Therefore, analysis of the active compounds from *Semen Torreyae* is a promising starting point to discover new anthelmintic drugs, but so far, none of its anthelmintic compounds have been identified.

The growth of parasitic nematodes involves multiple life stages, many of which are difficult to maintain in the laboratory. Thus, using parasitic nematodes as a model for anthelmintic discovery is typically expensive, labour-intensive and low-throughput (Katiki et al., 2011; Mathew, 2015). *Caenorhabditis elegans* (*C. elegans*) is an excellent model of parasitic nematodes for anthelmintic drug discovery and for elucidating the mechanism of action, owing to its similarity to parasitic species (Burns et al., 2015; Kaminsky et al., 2008; Weaver et al., 2017). It is a free-living nematode, which can easily be maintained in the laboratory and is amenable to high-throughput screening (Buckingham et al., 2014). For these reasons, we have chosen *C. elegans* as a model system to discover novel anthelmintic compounds from *Semen Torreyae* by assaying its locomotion.

In this study, we identified two anthelmintic compounds from *Semen Torreyae* using a bioassay-guided purification approach. A preliminary mechanism of action study was performed to determine the suitability of these compounds for further development as anthelmintics.

2. Material and methods

2.1. Chemicals and reagents

Acetone, *n*-hexane, ethyl acetate (EtOAc), methanol (MeOH), 2-propanol and acetonitrile (ACN), all of HPLC analytical grade, were purchased from Sigma-Aldrich (USA). Absolute ethanol was purchased from Fischer Chemicals (UK). Sterile ultra-pure water (MilliQ®) was produced by a water purification system (Milli-Q Reagent Water System, USA).

2.2. Plant material

Fresh *Semen Torreyae* was collected in Zhejiang Province, China in October 2013, followed by removal of their aril. Then the seeds were washed and dried in the sun. Dried *Semen Torreyae* botanical material (batch number 20131120) was purchased from Jinan Tongrentang Pharm. Co. Ltd. (Jinan, Shandong Province, China). The dried *Semen Torreyae* were oblong-ovate, with a length of 2-3.5 cm and a diameter of 1.3-2 cm. Their surface was sallow or light yellowish brown, with longitudinal wrinkles. The material was identified and authenticated according to the Chinese Pharmacopeia (2010 version) by a local botanist. A voucher specimen (L-ST-01) was deposited in the Department of Pharmaceutical Sciences, Shandong University, China.

2.3. Preparation of the extract

2.3.1. Small-scale preparation

The dried *Semen Torreyae* botanical material was ground to a fine powder. One gram of powder was transferred and divided over four 15 mL sterile Falcon tubes, and 10 mL of sterile water, absolute ethanol, hexane or acetone, respectively, was added to these tubes. Extraction was performed at ambient temperature with the aid of repeated vortexing and sonication (4 x 15 min over a 24 h period) in a sonicator water bath (Branson, USA). After 1 day, the tubes were centrifuged for 10 min at 3500 g (Hettich Rotanta 46R, C4810, Germany), and 1 mL aliquots of the supernatant were evaporated in a Savant SpeedVac Concentrator (SVC 200H, Stratech Scientific, UK), the dried residue of 1 mL of extract was re-dissolved in water (for the aqueous

extract) or DMSO (for the ethanol, hexane and acetone extracts) at a concentration of 40 mg/mL. The samples were stored at 4 °C until further testing.

2.3.2. Large-scale preparation

Fifty grams of powdered *Semen Torreyae* plant material was mixed with 0.5 L of ethanol and sonicated as mentioned in Section 2.3.1. The extract supernatant was decanted through filter paper (18.5 cm, Whatman Qualitative Filter Papers) and the filtrate was evaporated under reduced pressure on a Buchi® rotary evaporator. This process was repeated twice with the final sediment being centrifuged for 20 min at 1200 g at ambient temperature to recover as much extract as possible. To prepare for dry loading on a silica gel column, the dried extract (4.0 g) was dissolved in a small amount of the extraction solvent in the round-bottomed flask, and approximately 10 g of silica gel (63-200 µm) for every 10 g of dried extract was added to create a thick slurry mixture. This mixture was then evaporated until dry.

2.4. Anthelmintic activity test

2.4.1. Culture, maintenance and synchronization of *C. elegans* strains

The N2 wild-type *C. elegans* strain was maintained in our laboratory and used for anthelmintic screening during bioassay-guided purification. Other mutant strains were purchased from the *Caenorhabditis* Genetics Center (CGC, USA) to study the mechanism of action (see Tables 1 and 2). The worms were grown on Nematode Growth Medium (NGM) in Petri dishes containing a lawn of the bacterium *Escherichia coli* (*E. coli*) OP50 (Brenner, 1974). Synchronized populations were obtained by a modified alkaline bleaching method (Porta-de-la-Riva et al., 2012). Briefly, worm culture plates with eggs and egg-laying adults were washed with M9 buffer and incubated with freshly prepared bleaching solution, followed by three washes with M9. Then, 3.5 mL of worm suspension was added to 1.5 mL of alkaline hypochlorite solution (1 mL of bleach and 0.5 mL of 5 M NaOH). The suspension was shaken for 5 min, and 5.0 mL of a sucrose solution (60% w/v) was added. Later, 1 mL of M9 buffer was gently added to the mixture, followed by centrifugation at 300 g for 4 min at 20 °C (Hettich Rotanta 46R, C4810). After centrifugation, the eggs were

collected and washed five times with M9 buffer. Eggs in M9 were kept on a rotator at 20 °C overnight in the absence of light, to hatch into L1s. After 24 h of incubation, L1s were transferred onto a clean NGM plate seeded with an *E. coli* OP50 lawn and grown at 20 °C. After 48 h of incubation, young adult worms were washed off the plates with M9 buffer, before being used for assays with test samples.

2.4.2. Anthelmintic assay

The anthelmintic assay was carried out in a 96-well microplate (flat-bottomed, TPP Techno Plastic Products AG, Switzerland). Freshly cultivated young adult worms were collected in M9 buffer and adjusted to approximately 3000 larvae/mL. Then, 15 µL of this suspension (containing approximately 45 larvae) was added to each well of a 96-well microplate containing 184 µL of *E. coli* OP50 culture (O.D. 600 = 0.5). Subsequently, 1 µL of test sample (stock solution or dilution thereof in DMSO at different concentrations) was added to each well; DMSO (1 µL) was used in a separate well as a solvent control, and levamisole as a positive control. After mixing, the 96-well microplate was placed into a WMicrotracker apparatus (Phylumtech, Argentina) and incubated for 16 h at 20 °C. The movement of the worms in each well was measured every 30 min and recorded by the WMicrotracker. The percentage of the average movement over 16 h (except for the first h) of treated worms, compared with the DMSO control, was used to estimate the relative anthelmintic activity (Panda et al., 2017; Van Puyvelde et al., 2018). The same method was used to test the activity of active compounds on a panel of drug-resistant mutant strains (Table 1) and transient receptor potential (TRP)-channel mutant strains (Table 2) of *C. elegans*.

Table 1. Drug-resistant mutant strains of *C. elegans* used in this study

Strain	Genotype	Known resistance
DA1371	avr-14(ad1302)	ivermectin
JD596	avr-15(vu227)	ivermectin
DA1316	avr-14(ad1305) I; avr-15(vu227) glc-1(pk54) V	ivermectin
JD608	avr-14(ad1305) I; avr-15(vu227) glc-1(pk54) V	ivermectin
JD105	avr-15(ad1051) V	ivermectin
CB5	unc-7(e5)	ivermectin
CW129	unc-9(fc16)	ivermectin
ZM3087	unc-9(fc16) unc-7(e5)	ivermectin

CB904	unc-38(e264)	levamisole
ZZ37	unc-63(x37)	levamisole, DMPP
CB211	lev-1(e211)	levamisole
ZZ15	lev-8(x15)	levamisole
CB1072	unc-29(e1072)	levamisole
CB3474	ben-1(e1880)	benomyl
CB933	unc-17(e245)	aldicarb

DMPP, 1,1-dimethyl-4-phenylpiperazinium.

Table 2. TRP channel mutant strains of *C. elegans* used in this study

Strain	Gene name	Mutant allele
TQ233	trpa-1	ok999
RB2351	trpa-2	ok3189
CX4533	ocr-1	ok132
CX4544	ocr-2	ak47
LX950	ocr-4	vs137
VC1262	osm-9	ok1677
VC1242	cup-5	ok1698
VC160	trp-1	ok323
VC602	trp-2	gk298
VC1162	trp-3	ok1590
VC244	gtl-1	ok375
LH202	gtl-2	tm1463
TM2220	ced-11	tm2220
TM2946	gon-2	tm2946
TM3085	trpa-2	tm3085

2.5. Chromatography

2.5.1. Silica gel column chromatography

A 30 x 8 cm glass column was packed with 200 g silica gel (63-200 μ m) mixed with 500 mL of 100% hexane to a slurry consistency. The dried plant material adsorbed to silica gel was evenly distributed on the surface of the silica bed, and separated into 15 fractions using a step gradient consisting of 500 mL of each solvent mixture with increasing polarity. The column was initially eluted with hexane, followed by EtOAc and hexane mixtures (EtOAc:hexane, 25:75, 50:50, 75:25, 100:0). Then, the eluting solvent mixture was changed to MeOH:EtOAc (5:95, 10:90, 15:85, 20:80, 40:60, 60:40, 100:0) and finally to acetic acid:MeOH (5:95, 10:90, 15:85). All fractions were stored in a 4 °C cold room. One and a half mL of each of the 15 fractions was

aliquoted into 2 mL Eppendorf tubes and dried in the Savant SpeedVac Concentrator. A volume of DMSO was then added to dissolve the dried material to a final concentration of 20 mg/mL, and 1 μ L of this solution was used to test bioactivity against *C. elegans*. Fractions were then selected for further purification based on their activity against *C. elegans*. The cutoff for activity was set to 40% inhibition through all chromatography steps. Fraction F3 (1.2 g) was further divided into 60 subfractions (fr1-fr60) by flash silica gel chromatography on a column (4 $\times$ 40 cm) eluted with a solvent gradient hexane-EtOAc (100% hexane to 0% hexane). All the subfractions were tested against *C. elegans* at a final concentration of 80 μ g/mL. The most active fractions fr25 (89.5 mg) and fr30 (98.8 mg) were subjected to additional HPLC separation.

2.5.2. HPLC

An HPLC system (Shimadzu Corp., Japan) with a diode array detector (DAD) was used for HPLC purification.

A small aliquot of dried fr25 (0.5 mg) was dissolved in 1.0 mL of hexane and purified by preparative HPLC on a spherisorb cyano (CN) normal phase column (Waters, 4.6 $\times$ 250 mm, 5 μ m). Hexane and 2-propanol were used in a gradient elution mode. After the HPLC conditions were optimized, the gradient used was: starting with 100% hexane (5 min) and linearly increasing the 2-propanol from 0% to 8% over 5-30 min, from 8% to 40% over 30-40 min, followed by eluting with 40% 2-propanol for 10 min. The flow rate was set to 1.0 mL/min.

Also, a small aliquot of dried fr30 (1.2 mg) was dissolved in 2.0 mL of 50% ACN (in water) and purified on a C18 column (Sunfire®, 10 $\times$ 250 mm, 5 μ m). ACN and MilliQ® water, both with 0.05% trifluoroacetic acid (TFA) were used in a gradient elution mode. After the HPLC condition was optimized, the gradient used was: starting with 50% ACN (in water) (5 min) and linearly increasing the ACN from 50% to 78% over 5-8 min, from 78% to 88% over 8-21 min, from 88% to 100% over 21-22 min, followed by eluting with 100% ACN for 5 min. The flow rate was set to 4.0 mL/min. Eluate fractions were collected every minute. After they were dried in a SpeedVac Concentrator, 12 μ L of DMSO was added, and the solution was tested for

anthelmintic activity. The cutoff for activity was set to 40% inhibition. The active fractions were linked to the corresponding peaks by aligning the activity profile with the corresponding chromatogram. These peaks were then collected manually and dried, and their anthelmintic activity was verified. Then the active peaks were collected and analyzed for structural elucidation by gas chromatography-mass spectrometry (GC-MS), high resolution MS, and nuclear magnetic resonance (NMR). The total yield of compound 1 (C1) and 2 (C2) was 28.7 mg and 8.6 mg, respectively, from the initial 4.0 g of dry residue.

2.6. GC-MS, High resolution MS and NMR analysis

2.6.1. GC-MS analysis

C1 was analyzed on a gas chromatograph (Thermo Fisher Scientific Trace 1300 series) coupled with a mass spectrometer (Thermo Fisher Scientific ISQ series MS). The column used was a Restek RXi-5sil MS 20 m one with an internal diameter of 0.18 mm and a film thickness of 0.18 μ m. Helium was used as a carrier gas at a constant flow rate of 0.9 mL/min. The initial temperature of 40 °C was held for 2 min, then increased to 120 °C at a rate of 20 °C/min, to 200 °C at a rate of 10 °C/min, to 250 °C at a rate of 7 °C/min, and finally to 350 °C at a rate of 5 °C/min, which was held for 4 min. The spectrum corresponding to the largest peak in the chromatogram was searched against the NIST 14 MS library for identification. In addition, the identified compound was verified in terms of retention index, which was calculated using an external C7 to C40 alkane ladder.

2.6.2. UHPLC-TOF-MS analysis

LC-MS analysis was performed on an Agilent 1290 II Binary UHPLC system (UHPLC, Agilent Technologies, Santa Clara, CA, USA) coupled with an Agilent 6230 time-of-flight mass spectrometer (TOF-MS). Electrospray ionization (ESI) mass spectra were acquired in positive and negative mode. The mass analyzer was scanned from 100 to 1700 (m/z). The chromatographic separation was performed on an Agilent Extend-C₁₈ column (50 mm×2.1 mm, 1.8 μ m) with a constant temperature of 30 °C. The flow rate was 0.30 mL/min and the injection volume was 10 μ L with a concentration of 1 μ g/mL. The mobile phase consisted of 0.1% formic acid in water

(A) and 0.1% formic acid in ACN (B). A linear gradient was optimized as follows: 0-7 min, 5-95% B; 7-9 min, 95% B; 9-10 min, 95-5% B, and finally equilibration with 5% B for 3 min.

2.6.3. NMR spectroscopy

NMR spectra were recorded on a 600 MHz CryoFITNMR spectrometer (Bruker, Switzerland) in deuterated MeOH solution (C1) and a 400 MHz FT-NMR spectrometer (Bruker Avance 400, USA) in chloroform (C2) at the indicated temperatures. Chemical shifts are expressed in δ scale (ppm) using tetramethylsilane as an internal standard, and coupling constants J are expressed in Hz. The structures of purified compounds (C1 and C2) were elucidated by combining MS and NMR evidence. The ^1H and ^{13}C NMR spectral data are in agreement with earlier reports (Abe et al., 2002; Nam et al., 2005).

C1 : ^1H NMR (600 MHz, MeOD): δ 7.39-7.37 (d, J = 9.0 Hz, 2H), 7.11-7.09 (d, J = 9.0 Hz, 2H), 6.24-6.23 (d, J = 6.0 Hz, 1H), 6.06-6.01 (ddd, J = 6.0, 10.8, 16.8 Hz, 1H), 5.30-5.23 (m, 2H), 2.27 (s, 3H), 2.10 (s, 3H). ^{13}C NMR (151 MHz, MeOD): δ 171.7, 171.2, 152.0, 138.1, 137.6, 129.3, 122.9, 117.3, 77.1, 21.0, 20.9. HR-ESI-MS m/z $[\text{M}+\text{H}]^+$ 235.0955, calcd. 235.0964, $\text{C}_{13}\text{H}_{14}\text{O}_4$.

C2 : ^1H NMR (400 MHz, CDCl_3): δ 9.64 (s, 1H), 9.41 (s, 1H), 6.67 (t, J = 6.8 Hz, 1H), 3.41 (AB-pattern, J = 16.8 Hz, 2H), 2.36 (dd, J = 3.6 Hz, J = 45.6 Hz, 2H), 2.25 – 2.21 (m, 1H), 1.98 – 1.92 (m, 1H), 1.97 – 1.91 (m, 1H), 1.77 – 1.71 (m, 1H), 1.76 – 1.61 (m, 2H), 1.73 – 1.67 (m, 1H), 1.43 – 1.35 (m, 1H), 1.65 – 1.44 (m, 2H), 1.49 – 1.39 (m, 1H), 1.23 – 1.15 (m, 1H), 1.08 – 1.02 (m, 1H), 1.02 – 0.96 (m, 1H), 0.93 (s, 3H), 0.92 (s, 3H), 0.88 (s, 3H). ^{13}C NMR (101 MHz, CDCl_3): δ 196.94, 193.31, 160.47, 135.30, 57.64, 55.12, 52.83, 48.99, 41.97, 39.96, 39.50, 39.50, 35.91, 33.67, 33.66, 22.41, 21.86, 20.14, 18.68, 14.80. HR-ESI-MS m/z $[\text{M}+\text{H}]^+$ 319.2268, calcd. 319.2267, $\text{C}_{20}\text{H}_{30}\text{O}_3$.

2.7 Synergistic effect of active compounds from *Semen Torreyae*

The IC_{50} of each compound was used to determine the range of concentrations used for the synergy experiments. Each compound was tested both singly and in combination, at two 2-fold serial dilutions above and below the IC_{50} . The ratio of the

two tested compounds remained fixed across the dosing range. CalcuSyn software (Biosoft, USA) was used to evaluate the synergistic effects of the drug combination and generate combination index (CI) plots at the IC₅₀, IC₇₀, and IC₉₀ levels using the median-effect method (Chou and Talalay, 1984). A CI value < 1 indicates synergism, while a CI = 1 indicates additivity and a CI > 1 indicates antagonism (Grivicich et al., 2008).

2.8 Statistical analyses

All assays were carried out at least in duplicate and repeated at least once for confirmation. Data from dose-response experiments are represented as the percentage of inhibition and were analyzed with GraphPad Prism 6 software (San Diego, USA). A log (inhibitor) versus response non-linear fit was used to estimate the IC₅₀.

3. Results and discussion

3.1. Bioassay-guided purification and structure elucidation of active compounds

Semen Torreyae is a well-known traditional Chinese medicine for treating helminth diseases, but its anthelmintic components have not yet been identified. Therefore, a bioassay-guided isolation was performed with a *C. elegans* model. Even though *C. elegans* is a free-living nematode, it can still be used as an approximate model of parasitic worms thanks to its similarity to parasitic species (Buckingham et al., 2014). The motility of *C. elegans* is a useful index of the effects of anthelmintic drugs. In addition, the automated measurement of movement of worms in liquid media is well-suited for a readily scorable, phenotypic readout required for high-throughput screening (Buckingham et al., 2014; Buckingham and Sattelle, 2009; Bull et al., 2007). In our study, we used a WMicrotracker instrument to monitor the movement of worms continually. The quantitative data collected can easily be used for the potency evaluation of plant extracts and active compounds.

The ethanol and acetone extracts have significant anthelmintic activity against *C. elegans*, with the former showing the most potent activity (Fig. 1A). The strong effects elicited by crude extracts of *Semen Torreyae* further validate its ethnobotanical use as an anthelmintic in China. The ethanol extract of *Semen Torreyae* was selected

for subsequent large-scale extraction and bioassay-guided isolation of the active compounds.

Among the 15 fractions from preparative silica gel chromatography, F3 had the most potent activity (Fig. 1B) and was subjected to silica gel flash chromatography. The active subfractions (fr25 and fr30, Fig. 1C) were further purified by HPLC. Using a cutoff for the active peaks of 40% inhibition, bioassay-guided isolation led to two active peaks that were chromatographically pure (C1 and C2) (Fig. 2 and Fig. 3, their HRMS data and UV spectra were shown in Fig. S1-S4).

By a combination of GC-MS, HR-MS and NMR, the structure of compound C1 was elucidated as 1'-acetoxychavicol acetate (galangal acetate) and compound C2 was identified as (E)-8 β ,17-epoxylabd-12-ene-15,16-dial (miogadial). The potency of their anthelmintic activity was also determined; the IC₅₀ of galangal acetate and miogadial was $58.5 \pm 8.9 \mu\text{M}$ and $25.1 \pm 5.4 \mu\text{M}$, respectively (Fig. 4).

Although galangal acetate and miogadial are the main active compounds that we identified, it is possible that other low abundance active compounds went undetected in our bioassay. Nonetheless, the anthelmintic activity of our ethanol extract can almost completely be accounted for by the combined activity of the two isolated compounds. To the best of knowledge, this is the first time that galangal acetate and miogadial were isolated from this plant. Moreover, the anthelmintic activity of these two compounds has not yet been reported.

Fig. 1. Bioassay-guided purification of active anthelmintic compounds. (A) Anthelmintic activity (mean $\pm$ S.D., $n = 2$) of small-scale extracts (200 $\mu\text{g/mL}$) from four different solvents. (B) Anthelmintic activity (mean $\pm$ S.D., $n = 2$) of fractions from a preparative silica gel column (100 $\mu\text{g/mL}$) of *Semen Torreyae*. (C) Anthelmintic activity (mean $\pm$ S.D., $n = 2$) of subfractions from flash silica gel chromatography (80 $\mu\text{g/mL}$) of F3.

Fig. 2. HPLC chromatogram (254 nm) of fr25 resolved on a spherisorb CN normal phase column at a flow rate of 1.0 mL/min. The mobile phase was a mixture of hexane and 2-propanol; the gradient is indicated by a solid blue line.

Fig. 3. HPLC chromatogram (214 nm) of fr30 resolved on a C18 column at a flow rate of 4.0 mL/min. The mobile phase was a mixture of ACN and MilliQ® water, both with 0.05% TFA; the gradient is indicated by a solid blue line.

Fig. 4. Structures of active compounds isolated from *Semen Torreyae*.

Galangal acetate has been isolated from the seeds and rhizomes of ginger family plants, such as *Alpinia galangal*, and *Languas galangal* (Kojima-Yuasa et al., 2016). It was reported to possess a variety of pharmacological activities, such as antitumour, antileishmanial, antifungal, anti-obesity, anti-inflammatory and antioxidative activities *in vitro* and/or *in vivo* (Aziz et al., 2013; Latha et al., 2009; Lo et al., 2013; Ohnishi et al., 2012). Although galangal acetate has not been reported to have anthelmintic activity, an *Alpinia galangal* extract was reported to be moderately effective against human *Ascaris lubricoides* (Raj, 1975). The low general toxicity of galangal acetate is demonstrated by the general use of *Alpinia galangal* rhizome (rich in galangal acetate) as a seasoning for cooking in Southeast Asia (Warit et al., 2017; Yang and Eilerman, 1999). Moreover, there is a high survival rate of mice after consumption of galangal acetate at a dose of 100 mg/kg/d for 90 days (Mokkhasmit et al., 1971; Warit et al., 2017).

Galangal acetate is not stable in aqueous solutions and likely to undergo hydrolysis/isomerization reactions because of its allylic acetate structure (Yang and Eilerman, 1999). We also found that galangal acetate was not stable in a water:ACN mixture (containing 0.1% TFA) during HPLC purification. Therefore, a CN normal phase column with hexane and 2-propanol as mobile phases was used for its isolation. Hence, orally-administrated galangal acetate may be degraded to some extent in the gastrointestinal tract if it is used for anthelmintic purposes. In a botanical preparation, other components may stabilize galangal acetate during its passage through the gastrointestinal tract. For the pure compound, formulations could be developed to

stabilize it, or more stable analogues could be developed based on the galangal acetate pharmacophore.

Miogadial is a labdane-type terpenoid with an α,β -unsaturated 1,4-dialdehyde moiety, which has strong antimicrobial activities and inhibits human platelet aggregation and 5'-lipoxygenase *in vitro* (Iwasaki et al., 2009). The flower buds of myoga (*Zingiber mioga* Roscoe) (rich in miogadial) have been widely used as a spice and a pickle by the Japanese since ancient times (Abe et al., 2008; Abe et al., 2002). Although miogadial has not been reported to have anthelmintic activity, its close analogue, polygodial, has shown activity in a larval motility assay for the sheep nematode *Trichostrongylus colubriformis in vitro* (Lorimer et al., 1996). In our recent study, we found polygodial to exhibit potent anthelmintic activity against *C. elegans* with an EC_{50} of $13.1 \pm 5.3 \mu\text{M}$, and its α,β -unsaturated 1,4-dialdehyde moiety is essential for that activity (Liu et al., 2018). Considering that miogadial has a similar structure to polygodial and possesses an α,β -unsaturated 1,4-dialdehyde moiety as well, its anthelmintic activity is consistent with our previous findings.

3.2. Synergistic effect of galangal acetate and miogadial

Synergistic effects of medicinal plant components have been frequently reported. It is often assumed that synergy of ingredients in traditional medicinal plants will improve therapeutic efficacy (Kawakami et al., 2011; Wagner and Ulrich-Merzenich, 2009; Yang et al., 2014; Yuliana et al., 2011). Since the two isolated compounds from *Semen Torreya*: galangal acetate and miogadial, have obviously different structures, their modes of anthelmintic action are probably distinct, and the combination may be synergistic. This was evaluated using the median-effect method (Chou and Talalay, 1984). The combination of galangal acetate and miogadial indeed demonstrated synergy, at the IC_{50} , IC_{70} , and IC_{90} levels (Fig. 5). Drug development of natural products does not necessarily rely on the discovery of new structures from nature, but can also explore combinatorial drug regimes (Ulrich-Merzenich et al., 2010). Therefore, a combination of galangal acetate and miogadial could be attractive for achieving a better anthelmintic efficacy than the individual compounds; this can also

lower the toxicity of each component and reduce the risk of drug resistance in parasites.

Fig. 5. CI of evaluated IC₅₀, IC₇₀, and IC₉₀ for galangal acetate and miogadial (mean $\pm$ S.D., n = 2). CalcuSyn software (Biosoft, USA) was used to evaluate the synergistic effects of the drug combinations and generate CI plots at the IC₅₀, IC₇₀, and IC₉₀ levels. A CI value <1, = 1 and > 1 indicates synergism, additivity and antagonism, respectively.

3.3. Preliminary mechanism of action study of galangal acetate and miogadial

Understanding the mechanism of action of anthelmintics is of utmost importance for the further selection of effective agents to combat drug resistance in practice (Martin, 1997). Since the resistance to marketed anthelmintic drugs is widespread (Geurden et al., 2015; Laing et al., 2016), we screened galangal acetate and miogadial against a panel of *C. elegans* mutant strains (Table 1) resistant to currently-used anthelmintic drugs, to determine whether our compounds target a novel or established anthelmintic pathway. The cross-resistance results showed that galangal acetate and miogadial demonstrate similar activity against drug-resistant *C. elegans* strains as against the wild-type strain (Fig. 6). This implies that galangal acetate and miogadial are unlikely to target established anthelmintic targets, indicating that their mechanism of action probably differs from that of marketed drugs (ivermectin, levamisole, benomyl and aldicarb). Thus, galangal acetate and miogadial are promising anthelmintic hits, offering a good starting point to circumvent current drug resistance. Indeed,

anthelmintic compounds with novel modes of action can offer higher commercial returns in the pharmaceutical industry than those against established targets (Mathew, 2015).

Fig. 6. Anthelmintic activity (IC₅₀) of galangal acetate and miogadial on drug-resistant mutant *C. elegans* strains (mean $\pm$ S.D., n = 2). For list of strains, see Table 1.

Fig. 7. Anthelmintic activity (IC_{50}) of polygodial on TRP channel mutant *C. elegans* strains (mean $\pm$ S.D., $n = 2$). For list of strains, see Table 2.

Galangal acetate and miogadial were reported to activate transient receptor potential ankyrin 1 (TRPA1) channels in mammalian cells (Iwasaki et al., 2009; Narukawa et al., 2010). Moreover, TRP channels were proposed as possible anthelmintic targets (Bais et al., 2015; Bais and Greenberg, 2016). Thus, to further explore the possible mechanism of action of galangal acetate and miogadial, a panel of TRP channel mutant strains (Table 2) were tested to see whether their sensitivity to the two anthelmintic compounds is changed. The results showed that none of the TRP mutants was significantly resistant to galangal acetate or miogadial in comparison with wild type worms (Fig. 7). Therefore, an action on TRP channels appears to be unlikely as a mechanism of action of galangal acetate and miogadial. Thus, further research is necessary to unravel their mechanisms of action.

4. Conclusions

We provide scientific support for the traditional use of *Semen Torreyae* for treating intestinal parasites. Bioassay-guided isolation of anthelmintic compounds from *Semen Torreyae* yielded two active compounds: galangal acetate and miogadial, which demonstrate a synergistic effect against *C. elegans*. The anthelmintic activity of *Semen Torreyae* can mainly be ascribed to their combined activity. Preliminary mechanism of action studies of galangal acetate and miogadial suggest that they are unlikely to act on the targets of currently-used anthelmintic drugs, and an action on TRP channels also appears to be ruled out. Their mechanisms of action remain to be elucidated. Overall, galangal acetate and miogadial could be promising anthelmintic hits worth further investigation.

Acknowledgement

We thank Dr. Kurt Boonen for LC-MS analysis, and An Vandoren for GC-MS analysis. Maoxuan Liu was supported by a Chinese Scholarship Council doctoral fellowship (201406220169), and Cedrick Veryser by an FWO (Ph.D. Fellowship of the Research Foundation – Flanders) fellowship. Walter Luyten largely supported

himself. Some *C. elegans* strains were obtained from the CGC, which is funded by NIH Office of Research Infrastructure Programs (P40 OD010440). Other *C. elegans* strains were provided by the MITANI Lab through the National Bio-Resource Project of the MEXT, Japan. The Hercules Foundation is acknowledged for supporting the purchase of a 400 MHz NMR spectrometer through project AKUL1311.

Declaration of interest

Conflicts of interest

none.

Author contributions

Study conception and design: WL, ML. Acquisition of data: ML, CV, JL. Analysis and interpretation of data: ML, CV, JL, TW, WMDB, ZJ, WL. Drafting of manuscript: WL, ML. Critical revision: CV, JL, TW, WMDB, ZJ, WL.

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