Accepted Manuscript

Bioassay-guided isolation of three anthelmintic compounds from *Warburgia ugandensis* Sprague subspecies *ugandensis*, and the mechanism of action of polygodial

Maoxuan Liu, Purity Kipanga, Anh Hung Mai, Ineke Dhondt, Bart P. Braeckman, Wim De Borggraeve, Walter Luyten

PII: S0020-7519(18)30151-6
DOI: <https://doi.org/10.1016/j.ijpara.2017.11.009>
Reference: PARA 4078

To appear in: *International Journal for Parasitology*

Received Date: 7 October 2017
Revised Date: 27 November 2017
Accepted Date: 27 November 2017

Please cite this article as: Liu, M., Kipanga, P., Mai, A.H., Dhondt, I., Braeckman, B.P., De Borggraeve, W., Luyten, W., Bioassay-guided isolation of three anthelmintic compounds from *Warburgia ugandensis* Sprague subspecies *ugandensis*, and the mechanism of action of polygodial, *International Journal for Parasitology* (2018), doi: <https://doi.org/10.1016/j.ijpara.2017.11.009>

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Bioassay-guided isolation of three anthelmintic compounds from *Warburgia ugandensis* Sprague subspecies *ugandensis*, and the mechanism of action of polygodial

Maoxuan Liu^{a,d,*}, Purity Kipanga^a, Anh Hung Mai^{b,e}, Ineke Dhondt^c, Bart P. Braeckman^c, Wim De Borggraeve^b, Walter Luyten^d

^a *Faculty of Pharmaceutical Sciences, KU Leuven, Herestraat 49, Box 921, 3000 Leuven, Belgium*

^b *Department of Chemistry, Molecular Design and Synthesis, KU Leuven, Celestijnenlaan 200F, box 2404, 3000 Leuven, Belgium*

^c *Department of Biology, Faculty of Sciences, Ghent University, K.L. Ledeganckstraat 35, 9000 Gent, Belgium*

^d *Department of Biology, Animal Physiology and Neurobiology Section, KU Leuven, Naamsestraat 59, Box 2465, 3000 Leuven, Belgium*

^e *Present address: PolymerExpert, Pessac, France*

*Corresponding author.

E-mail address: maoxuan.liu@kuleuven.be

18 **Abstract**

19 Parasitic helminths continue to pose problems in human and veterinary medicine, as well as in
20 agriculture. Resistance to current anthelmintics has prompted the search for new drugs.
21 Anthelmintic metabolites from medicinal plants could be good anthelmintic drug candidates.
22 However, the compounds active against nematodes have not been identified in most medicinal
23 plants with anthelmintic activity. In this study, we aimed to identify the active compounds against
24 helminths in *Warburgia ugandensis* Sprague subspecies *ugandensis* (Canellaceae) and study the
25 underlying mechanism of action. A bioassay-guided isolation of anthelmintic compounds from the
26 plant was performed using a *Caenorhabditis elegans* test model with a WMicrotracker instrument
27 to monitor motility. Three active compounds were purified and identified by nuclear magnetic
28 resonance and high resolution MS: warburganal (IC₅₀: 28.2 ± 8.6 µM), polygodial (IC₅₀: 13.1 ± 5.3
29 µM) and alpha-linolenic acid (ALA, IC₅₀: 70.1 ± 17.5 µM). A checkerboard assay for warburganal
30 and ALA as well as polygodial and ALA showed a fractional inhibitory concentration index of 0.41
31 and 0.37, respectively, suggesting that polygodial and ALA, as well as warburganal and ALA, have
32 a synergistic effect against nematodes. A preliminary structure-activity relationship study for
33 polygodial showed that the α,β-unsaturated 1,4-dialdehyde structural motif is essential for the
34 potent activity. None of a panel of *C. elegans* mutant strains, resistant against major anthelmintic
35 drug classes, showed significant resistance to polygodial, implying that polygodial may block *C.*
36 *elegans* motility through a mechanism which differs from that of currently marketed drugs. Further
37 measurements showed that polygodial inhibits mitochondrial ATP synthesis of *C. elegans* in a
38 dose-dependent manner (IC₅₀: 1.8 ± 1.0 µM). Therefore, we believe that the underlying mechanism
39 of action of polygodial is probably inhibition of mitochondrial ATP synthesis. In conclusion,
40 polygodial could be a promising anthelmintic drug candidate worth considering for further
41 development.

42 **Keywords:** Anthelmintic, *Warburgia ugandensis*, *Caenorhabditis elegans*, Polygodial,
43 Mitochondrial ATP synthesis

1. Introduction

Parasitic helminths continue to pose problems in human and veterinary medicine, as well as in agriculture (Taman and Azab, 2014; Loukas et al., 2016). It is estimated that approximately one-third of humans (more than 2 billion people), especially those in developing regions, are infected with one or more nematodes (Rana and Misra-Bhattacharya, 2013). Intestinal nematode parasites such as *Ascaris lumbricoides* and *Trichuris trichiura* can produce a wide range of symptoms including intestinal manifestations, general malaise and weakness, which may affect working and learning capacities and impair physical growth (Norhayati et al., 2003; Bajin Ba Ndob et al., 2016; Brindley and Loukas, 2017). Additionally, parasitic nematodes are a significant cause of economic loss in livestock and crop industries around the world. For example, in the USA alone, nematodes cost the livestock industry an estimated US \$2 billion per year in lost productivity and increased operating expenses (Li et al., 2011).

In the absence of vaccines for intestinal nematodes, control of infections relies mainly on chemotherapy. However, anthelmintic resistance has been widely reported in livestock and occasionally in human parasites. It may be only a matter of time before this phenomenon becomes common in parasites of humans (Bachaya et al., 2009; Kaewintajuk et al., 2010; Taman and Azab, 2014; Geurden et al., 2015; Laing et al., 2016). Thus, the need for new anthelmintic compounds with novel mechanisms of action is clear (Epe and Kaminsky, 2013).

Medicinal plants hold great promise as a source of effective treatments as anthelmintic agents. They have been used traditionally due to their efficacy and safety (Romero-Benavides et al., 2017). Anthelmintic metabolites from these medicinal plants could be good anthelmintic drug candidates (Kumarasingha et al., 2016). A few natural products from medicinal plants have already been identified using both in vitro and in vivo models of *Trichuris muris* and *Schistosoma mansoni* (Edwards et al., 2015; Wangchuk et al., 2016a, b). However, in most medicinal plants the compounds active against nematodes have not yet been identified.

The free-living nematode *Caenorhabditis elegans* was demonstrated to be a useful model system for the discovery of new anthelmintic drugs, and for characterizing their mechanisms of action or resistance (Holden-Dye and Walker, 2014; Burns et al., 2015; Kumarasingha et al., 2016; Salas et al., 2016; Weaver et al., 2017). Due to its easy maintenance in the laboratory, small size and short generation time, *C. elegans* is attractive for evaluating the anthelmintic effect of crude plant extracts or pure compound without the need of host infection experiments (Katiki et al., 2011; Kumarasingha et al., 2014; Pina-Vazquez et al., 2017). Therefore, we chose *C. elegans* as a model system to discover novel anthelmintic compounds from medicinal plants by assaying its locomotion (Buckingham et al., 2014; Holden-Dye and Walker, 2014).

We selected some medicinal plants used traditionally for treating parasitic diseases, and screened their anthelmintic activity in the *C. elegans* model. One plant, *Warburgia ugandensis* Sprague subspecies *ugandensis* (Canellaceae), showed promising activity. *Warburgia ugandensis* is widely used as a medicinal plant in tropical and subtropical Africa (especially in Kenya and Uganda). The species has become endangered due to its popularity in traditional medicine, which has led to unsustainable bark harvesting. The bark is usually chewed and the juice swallowed for the treatment of stomach ache, cough, fever, toothache, venereal diseases, constipation, weak joints and general body pain (Leonard and Viljoen, 2015). In Kenya, the hot decoction of the bark or leaves from *W. ugandensis* (subsp. *ugandensis*) is also taken orally to treat helminthiasis and as an emetic. In addition, in veterinary medicine the hot decoction of the bark or leaves is used for the treatment of ectoparasitism, heartwater, black quarter and trypanosomiasis (Nanyingi et al., 2008; Maroyi, 2014). However, the anthelmintic compounds in this medicinal plant have not been identified.

In this study, a bioassay-guided isolation of anthelmintic compounds from *W. ugandensis* (subsp. *ugandensis*) was performed using a *C. elegans* testing model. The underlying anthelmintic mechanism of action of the most potent compound isolated from the plant was also investigated.

95 2. Materials and methods

96 2.1. Chemicals and reagents

97 Acetone, n-hexane, ethyl acetate (EtOAc), methanol (MeOH) and acetonitrile (ACN), all of
98 HPLC analytical grade, were purchased from Sigma-Aldrich (USA). Absolute ethanol was
99 purchased from Fischer Chemicals (UK). Sterile ultra-pure water (MilliQ®) was produced by a
100 water purification system (Milli-Q Reagent Water System, MA, USA). DMEM (D6429) was
101 purchased from Sigma-Aldrich. Ultra-Pure ADP was purchased from Cell Technology, Inc. (USA).
102 Other reagents were purchased from Sigma-Aldrich. Analogues Sigma1-2 were purchased from
103 Sigma-Aldrich, while mukaadial and Specs1-11 were from Specs (The Netherlands). Ugandenial A
104 was prepared from leaves of *W. ugandensis*, while drimendiol was semisynthesized from
105 polygodial. Since the structures of these compounds are similar to polygodial, they were selected
106 for activity testing to help elucidate the active pharmacophore of polygodial, and to try to find more
107 potent compounds.

109 2.2. Plant material

110 Six indigenous medicinal plants were chosen based on their ethnopharmacological use in
111 Kenya for the treatment of different infectious diseases. Fresh leaves of the medicinal plants were
112 collected from the Kakamega forest, an expansive 238 square km tropical rainforest located in
113 western Kenya. The leaves were identified by a senior botanist and then transported in sacks to the
114 Egerton University Biotechnology Laboratories (Kenya) where they were air-dried at ambient
115 temperature with frequent turning until they were completely dried. A standard electric miller was
116 used to mill the leaves and twigs (when present) into powder. They were packed in brown paper
117 bags, certified by Kenyan authorities and exported to Belgium. The voucher specimen of the leaves
118 of *W. ugandensis* (NWB237), *Albizia gummifera* (PWM25), *Zanthoxylum chalybeum* (STK102),

119 *Zanthoxylum gilleti* (STK101), *Turrea holstii* (STK257), *Toddalia asiatica* (PWM 196) were
120 deposited at the Egerton University Herbarium.

121

122 2.3. Preparation of the extract

123 2.3.1. Small-scale preparation

124 The dried raw botanical material was ground to a fine powder. One gram of powder was
125 transferred into each of four 15 mL sterile Falcon tubes, and 10 mL of sterile water, absolute
126 ethanol, hexane or acetone were added, respectively. Extraction was performed at ambient
127 temperature with the aid of repeated vortexing and sonication (4 x 15 min over a 24 h period) in a
128 sonicator water bath (Branson, USA). After 1 day, the tubes were centrifuged for 10 min at 3500 g
129 (Hettich Rotanta 46R, C4810, Germany), and the supernatant transferred in 1 mL aliquots to 1.5 mL
130 Eppendorf tubes. After evaporation of the solvent in a Savant SpeedVac Concentrator (SVC 200H,
131 Stratech Scientific, London, UK), the dried residue of 1 mL of extract was re-dissolved in water (for
132 the aqueous extract) or DMSO for the ethanol, hexane and acetone extracts at a concentration of 40
133 mg/mL. The samples were stored at 4 °C until further testing.

134

135 2.3.2. Large-scale preparation

136 Fifty grams of powdered *Warburgia ugandensis* plant material were mixed with 0.5 L of
137 ethanol in a screw-capped glass bottle, shaken and sonicated as mentioned in Section 2.3.1. The
138 extract supernatant was filtered through a filter paper (18.5 cm, Whatman Qualitative Filter Papers)
139 and evaporated under reduced pressure in a pre-weighed, round-bottomed flask in a Buchi® rotary
140 evaporator. This process was repeated twice with the final sediment being centrifuged for 20 min at
141 1200 g at ambient temperature to recover as much extract as possible. To prepare for dry loading on
142 a silica gel column, the dried extract was dissolved in the round-bottomed flask with a small amount

of the solvent used to prepare the extract, and approximately 10 g of silica gel (63-200 μ m) for every 10 g of dried extract were added to create a thick slurry mixture. This mixture was then evaporated until dry. The extract-silica gel mixture was then scraped off the flask and stored in a 50 mL conical plastic tube with a screwcap.

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, 2). The worms were grown on Nematode Growth Medium (NGM) in Petri dishes containing a lawn of the bacterium *Escherichia 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 were 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) were 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 from 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 μ L of this suspension (containing approximately 45 larvae) were added to each well of a 96-well microplate containing 184 μ L of *E. coli* OP50 culture (O.D. 600 = 0.5). Subsequently, 1 μ L of test sample (stock solution or dilution thereof in DMSO at different concentrations) was added to each well; DMSO (1 μ L) was used in a separate well as a solvent control. After mixing, the 96-well microplate was placed into a WMicrotracker (Phylumtech, Argentina) apparatus and incubated for 16 h at 20 °C. The movement of 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 test samples, compared with the DMSO control, was used to estimate the relative anthelmintic activity. 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*.

2.5. Chromatography

2.5.1. Preparative silica gel column chromatography

A 30 cm by 8 cm glass column was packed with 200 g of silica gel (63-200 μ m), mixed with 500 mL of 100% hexane to a slurry consistency. Once the silica gel had settled, 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 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 labeled 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*.

2.5.2. HPLC

One mL of a dried-down active fraction of a plant extract was dissolved in 1 mL of 50% ACN (in water) mixture. The solution was then centrifuged for 30 s and filtered through a CHROMAFIL® Xtra H-PTFE filter (pore size 0.45 μ m, filter 13 mm, MACHEREY-NAGEL, Germany), topped up to 2 mL with 50% ACN (in water), and injected into a HPLC system (Shimadzu Corp., Japan) with a diode array detector (DAD) and a C18 column (Sunfire®, 10 x 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 the 50% ACN (in water) and linearly increasing the ACN from 50% to 80% over 5-25 min, and from 80% to 83% over 25-44 min, and from 83% to 100% over 44-45 min, followed by eluting with 100% ACN for 5 min. The flow rate was 4.0 mL/min. Eluate fractions were collected every minute. After they were dried down by the SpeedVac Concentrator, 12 μ L of DMSO were added and the solution was tested for anthelmintic activity. The cutoff for activity was set to 40% inhibition. The active fractions were associated with the corresponding peaks in the chromatogram. These peaks were then collected manually and dried, and their anthelmintic activity was verified. Then the active peaks were collected for high resolution MS and nuclear magnetic resonance (NMR) analysis for structural elucidation.

2.6. High resolution MS and NMR

High resolution MS were acquired on a quadrupole orthogonal acceleration time-of-flight mass spectrometer (Synapt G2 HDMS, Waters, USA). Samples were infused at 3 μ L/min and spectra were obtained in positive (or negative) ionization mode with a resolution of 15,000 (FWHM) using leucine-enkephalin as lock mass. NMR spectra were recorded on a 600 MHz FT-NMR spectrometer, in deuterated chloroform and at indicated temperatures; chemical shifts are expressed in δ scale (ppm) using tetramethylsilane as an internal standard, and coupling constants J are in Hz. The structures of purified compounds (C1-3) were elucidated by combining MS and NMR evidence. The ^1H and ^{13}C NMR spectral data were compared with earlier reports (Gunstone et al., 1977; Urones et al., 1994; Lee et al., 2004; Just et al., 2015) of the inferred compounds.

C1: ^1H NMR (600 MHz, CDCl_3 , 288K): δ 9.73 (s, 1H), 9.42 (s, 1H), 7.28 (dd, $J = 5.0$ Hz, 2.4 Hz, 1H), 4.10 (brs, 1H), 2.59 (dt, $J = 20.9$ Hz, 5.0 Hz, 1H), 2.35 (ddd, $J = 20.9$ Hz, 11.9 Hz, 2.4 Hz, 1H), 1.89 (dd, $J = 11.9$ Hz, 5.0 Hz, 1H), 1.71-1.66 (m, 2H), 1.52-1.49 (m, 2H), 1.46-1.42 (m, 1H), 1.28-1.23 (m, 1H), 1.10 (s, 3H), 0.99 (s, 3H), 0.95 (s, 3H). ^{13}C NMR (150 MHz, CDCl_3 , 288K): δ 202.3, 192.8, 157.7, 140.2, 77.6, 41.7, 41.4, 41.2, 33.1, 33.0, 31.1, 25.9, 22.1, 17.7, 17.1. HR-ESI-MS m/z $[\text{M}+\text{H}]^+$ 249.1493, calcd. 249.1496, $\text{C}_{15}\text{H}_{22}\text{O}_3$.

C2: ^1H NMR (400 MHz, CDCl_3 , 298K): δ 9.53 (d, $J = 4.4$ Hz, 1H), 9.46 (s, 1H), 7.12 (m, 1H), 2.83 (m, 1H), 2.51 (m, 1H), 2.31 (m, 1H), 1.84 (m, 1H), 1.59-1.45 (complex m, 3H), 1.38 (td, $J = 12.9$, 4.5 Hz, 1H), 1.30-1.19 (complex m, 2H), 0.96 (s, 3H), 0.95 (s, 3H), 0.92 (s, 3H). ^{13}C NMR (100 MHz, CDCl_3 , 298K): δ 201.9, 193.2, 154.2, 138.3, 60.3, 49.0, 41.7, 39.6, 36.9, 33.14, 33.13, 25.2, 22.0, 18.0, 15.3. HR-ESI-MS m/z $[\text{M}+\text{H}]^+$ 235.1700, calcd. 235.1693, $\text{C}_{15}\text{H}_{22}\text{O}_2$.

C3 : ^1H NMR (600 MHz, CDCl_3 , 298K): δ 5.42-5.30 (m, 6H), 2.81 (t, $J = 6.2$ Hz, 4H), 2.35 (t, $J = 7.5$ Hz, 2H), 2.11-2.03 (m, 4H), 1.65-1.61 (m, 2H), 1.36-1.28 (m, 8H), 0.98 (t, $J = 7.5$ Hz, 3H). ^{13}C NMR (150 MHz, CDCl_3 , 298K): δ 179.5, 131.9, 130.2, 128.2, 128.2, 127.7, 127.0, 33.9, 29.5, 29.2, 29.1, 29.0, 27.2, 25.6, 25.5, 24.6, 20.5, 14.3. HR-ESI-MS m/z $[\text{M}+\text{H}]^+$ 277.2175, calcd. 277.2173, $\text{C}_{18}\text{H}_{30}\text{O}_2$.

243

244 *2.7. Cytotoxicity assay*

245 Cell viability was determined using a Methylthiazolyldiphenyl-tetrazolium bromide (MTT)
246 assay (Gerlier and Thomasset, 1986). Two non-tumoural cell lines, HEK 293 and RAW 264.7
247 (purchased from ATCC, USA), were maintained in DMEM, supplemented with penicillin (100
248 U/mL), streptomycin (100 µg/mL) and 10% FBS, at 37 °C and in a humidified incubator with a 5%
249 CO₂ atmosphere. The cells were plated at a density of 7,500 cells per well (100 µL) in a 96-well
250 plate and cultured for 24 h. The cells were subsequently exposed to various concentrations of
251 warburganal, alpha-linolenic acid (ALA) and polygodial. The plates were incubated for 24 h and
252 cell viability was measured by adding 20 µL of MTT dye (5 mg/mL in PBS) per well. The plates
253 were incubated for a further 3.5 h at 37 °C in a humidified incubator containing 5% CO₂. Formosan
254 crystals in each well were dissolved in 150 µL of DMSO, and absorbance was read at 590 nm with
255 a reference filter of 620 nm. The absorption values were expressed as the cell viability (%)
256 compared with the DMSO control group (set at 100%). β-Lapachone (40 µM) was used as a
257 positive (cytotoxic) control in this assay.

258

259 *2.8. Synergistic effect of active compounds using checkboard assay*

260 The synergistic effect of active compounds from the plant was tested using a checkerboard
261 assay (Orhan et al., 2005). The Fractional Inhibitory Concentration (FIC) index value was used to
262 quantify the interactions between the compounds being tested. $\Sigma FIC = FIC_A + FIC_B = (C_A/MIC_A) +$
263 (C_B/MIC_B) , where MIC_A and MIC_B are the minimum inhibitory concentrations (MICs) of
264 compounds A and B alone, respectively, and C_A and C_B are the concentrations of the compounds in
265 combination, respectively, in all of the wells corresponding to an MIC. $\Sigma FIC \leq 0.5$ was interpreted
266 as synergy while $\Sigma FIC > 4.0$ was interpreted as antagonism and $0.5 < \Sigma FIC < 4.0$ was interpreted
267 as no significant interaction (Odds, 2003).

268

269 *2.9. Mitochondrial ATP production measurement*270 *2.8.1. Preparation of C. elegans mitochondria*

271 Mitochondria were isolated as described before (Kayser et al., 2001; Brys et al., 2010).
272 Briefly, approximately 20,000 age-synchronized young adult worms (N2 strain) were harvested
273 from Petri plates and washed with MilliQ® water to remove the M9 buffer, and finally suspended
274 in mitochondrial isolation buffer (MSME) according to Kayser et al. (2001). All subsequent
275 treatments were performed at 4 °C. The worm suspension was transferred to a sampling tube and
276 homogenized using an IKA Ultraturrax rotor-stator mixer (IKA Werke, Staufen, Germany). Next,
277 an equal volume of MSME containing 0.4% BSA was added and the suspension was thoroughly
278 mixed by inversion, and centrifuged for 5 min at 400 g to remove large debris and nuclei. The
279 supernatant was transferred to a fresh centrifuge tube and centrifugation was repeated. The resulting
280 supernatant containing crude mitochondria was centrifuged for 5 min at 4500 g and centrifugation
281 was repeated once. The resulting mitochondrial pellet was resuspended in MSME. The protein
282 content was measured using the bicinchoninic acid (BCA) assay (Walker, 1994); 20,000 adult
283 worms yielded ~65 µg of mitochondrial protein.

284

285 *2.8.2. Quantification of mitochondrial ATP synthesis*

286 ATP synthesis was determined using the Roche (Switzerland) ATP Bioluminescence Assay
287 Kit CLS II as described (Brys et al., 2010). Approximately 5 ng (on a protein basis) of freshly
288 isolated mitochondria were added to the wells of a white microtitre plate containing 96 µL of
289 incubation medium, 50 µL of luciferase reagent (one bottle was dissolved in 5 mL of MilliQ®
290 water) and 50 µL of substrate/ADP mix (final concentrations in the well: pyruvate 1 mM, malate 1
291 mM, ADP 100 µM). The incubation medium was modified Oxphos buffer (100 mM KCl, 50 mM
292 MOPS, 1 mM EGTA, 5 mM K₂HPO₄). The emitted light was measured for 45 min in a Wallac

Victor2 Multilabel Counter (PerkinElmer, USA). Oligomycin (4 µg/mL) was used as a positive control in this assay. The percentage of the average ATP production compared with the DMSO control was used to estimate the relative inhibition activity.

296

2.10. Morphological study of polygodial-treated worms

2.10.1. Mitochondrial morphology

Mitochondrial morphology was studied using the reporter strain *jrIs5[unc-119(+)* *rps-Op::mIs::Dendra2*] constructed in the laboratory of Prof. Braeckman (Ghent University, Belgium) (Castelein et al., 2014). Confocal images were recorded using a Nikon TiE-C2 confocal laser scanning microscope. Synchronised young adult Dendra2 transgenic worms were treated with 0.5% DMSO (control) or polygodial (20 µM, 40 µM and 100 µM) for 3 h, and then immobilized on 5% agarose pads using 10 mM levamisole. Images of the mid-body of the worms were collected using a 488-nm solid state laser for excitation combined with a 525/50 nm bandpass emission filter and a 40x CFI Plan Apochromat objective (NA 1.25, water immersion). Detection parameters (laserpower, high voltage and offset) were maintained as constant. Images were made of at least three worms from cultures treated with 0.5% DMSO (control) and polygodial (20 µM, 40 µM and 100 µM).

310

2.10.2. Scanning electron microscopy (SEM)

Synchronized young adult *C. elegans* worms were treated with 100 µM polygodial for 16 h and then fixed in 2.5% (w/v) glutaraldehyde in PBS for 24 h at 22-24 °C. After fixation, the worms were washed three times in PBS and stored in 70% ethanol at 22-24 °C until use. Subsequently, the worms were placed in a mixture (1:1) of 70% ethanol and dimethoxymethane (DMM) for 5 min for dehydration, then transferred to 100% DMM for 20 min and critical point dried using liquid CO₂

316

with a CPD 030 critical point dryer (BAL-TEC AG, Lichtenstein). Critical point dried worms were mounted on aluminium stubs, sputter-coated with gold, and observed under a JEOL JSM-6360 scanning electron microscope (JEOL Ltd., Japan) (Dirks-Mulder et al., 2017).

2.11. Statistical analyses

All assays were carried out at least in duplicate and repeated at least once for confirmation. Data from dose-response experiments were represented as the percentage of inhibition, and analyzed with GraphPad Prism 6 software (San Diego, CA, USA). A log (inhibitor) versus response non-linear fit was used to estimate the 50% inhibitory and cytotoxic concentrations (IC_{50} and CC_{50}). Statistical analysis was done using one-way ANOVA with Dunnett's test to assess the test samples versus DMSO (N2) control.

3. Results

3.1. Bioassay-guided purification and elucidation of active compound structures

The 24 samples prepared by small-scale extraction of six medicinal plants in four different solvents were tested for anthelmintic activity at the same concentration (200 μ g/mL). The results showed that ethanol and acetone extracts of *W. ugandensis* have quite strong anthelmintic activity in the WMicrotracker assay, with the ethanol extract showing the most potent activity among the four extracts (Fig. 1A). In addition, ethanol extracts of *Z. chalybeum* and *Z. gilleti* showed slight activity. Thus, the ethanol extract of *W. ugandensis* was selected for subsequent large-scale extraction and bioassay guided isolation of active compounds. From 50 g of plant material, 6.7 g of dry residue were obtained, and separated by preparative silica gel column chromatography using step-wise elution to yield 15 fractions (F1-15, see Section 2.5.1. for eluent composition). Their anthelmintic activity was tested at the same concentration (100 μ g/mL). The results suggested that

F3 (eluted by EtOAc:hexane (1:1); yield: 1.8 g) had the most potent activity (Fig. 1B). Aliquots (~4 mg) of F3 were subjected to HPLC purification, and the subfractions were collected and tested. Using a cutoff for the active peaks of 40% inhibition, three peaks (C1, C2, C3) were identified as active (Fig 1C, D). These three peaks were collected and analyzed by high resolution MS and NMR.

The isolated compounds were identified following analysis of their spectroscopic data and by comparison with published data. Their structures were elucidated as warburganal (C1) (Urones et al., 1994; Lee et al., 2004), polygodial (C2) (Just et al., 2015) and ALA (C3) (Gunstone et al., 1977) (Fig. 2). The total yield of warburganal, polygodial and ALA was 48.9 mg, 7.5 mg and 13.4 mg, respectively, from the 6.7 g of dry residue. The potency of their anthelmintic activity was determined by a dose-response curve, and expressed as the IC_{50} . The IC_{50} of warburganal, polygodial and ALA were $28.2 \pm 8.6 \mu M$, $13.1 \pm 5.3 \mu M$ and $70.1 \pm 17.5 \mu M$, respectively.

3.2. Cytotoxicity of compounds isolated from the plant

Understanding the cellular toxicity is important in drug discovery, and eukaryotic cell cultures are accepted as the model system of choice to obtain a first approximation of toxicity (Atterwill and Steele, 1987). To examine the potential toxicity of active compounds that were isolated, the cytotoxicity of warburganal, ALA and polygodial was tested on two non-tumoural cell lines, HEK 293 and RAW 264.7. The results showed that ALA has almost no cytotoxicity on the two cell lines, while warburganal and polygodial have a CC_{50} of 45~65 μM (Table 3). The selectivity index (the ratio of IC_{50} on *C. elegans* to CC_{50} on two mammalian cells) for warburganal and polygodial is 1.7 and 4.6, respectively.

3.3. Synergistic effect of warburganal, polygodial, and ALA

Two of the active compounds (warburganal and polygodial) have similar structures (drimane sesquiterpenoids), while ALA belongs to a completely different compound class (fatty acid). Thus, a checkerboard assay was used to test whether any pair of compounds had a synergistic effect against *C. elegans*. The MIC values of warburganal (A), polygodial (B) and ALA (C) were significantly higher than their IC₅₀ values: $254.3 \pm 14.7 \mu\text{M}$, $91.5 \pm 10.0 \mu\text{M}$ and $309.8 \pm 28.7 \mu\text{M}$, respectively. The checkerboard result indicated that the combination of warburganal (65.5 μM) + ALA (48.4 μM), polygodial (26.8 μM) + ALA (24.2 μM), and warburganal (65.5 μM) + polygodial (53.6 μM) have the same anthelmintic effect as MIC_A, MIC_B or MIC_C. Therefore, the FIC for these pairs are 0.84, 0.41 and 0.37, suggesting that both warburganal and polygodial are synergistic with ALA, but that there is no synergy between warburganal and polygodial.

3.4. Preliminary structure-activity relationship of polygodial

Since polygodial showed the most potent activity against *C. elegans* among the three active plant compounds, 18 analogues of polygodial were tested for anthelmintic activity (Table 4) to better understand the structural elements that have an impact on its bioactivity. Most of the analogues contain the drimane sesquiterpene skeleton. The results showed that warburganal, polygodial and mukaadial have potent activity (IC₅₀: 10~30 μM) (Table 4). Drimenol is less potent while Sigma1 and Specs4 are almost inactive. This suggests that the α,β -unsaturated 1,4-dialdehyde of polygodial is essential for potent activity.

3.5. Mechanism of action study of polygodial

To study the mechanism of action of polygodial, the anthelmintic activity was tested on a panel of drug-resistant *C. elegans* mutant strains to see whether polygodial has a similar mechanism of action as marketed drugs. Polygodial showed similar activity against ivermectin-, benomyl-, aldicarb- and levamisole-resistant *C. elegans* strains as against the wild-type (N2) strain (Fig. 3A).

This result suggests that the mechanism of action of polygodial probably differs from that of these marketed drugs (ivermectin, benomyl, aldicarb and levamisole).

Polygodial was reported to activate TRPA1 in mammalian cells (Escalera et al., 2008). Thus, a panel of TRP channel mutant strains was tested to see whether their sensitivity to polygodial was changed. The results showed that none of the mutants is significantly resistant to polygodial in comparison with N2 wild type worms (Fig. 3B). Only VC602 showed a ~two-fold increased resistance to polygodial. However, this slight increase cannot support the conclusion that the target of polygodial is the *trp-2* gene product.

It has been reported that polygodial can inhibit mitochondrial ATP synthesis of *Saccharomyces cerevisiae* (Lunde and Kubo, 2000) and mammals (Castelli et al., 2005). Therefore, the effect of polygodial on mitochondrial ATP synthesis of *C. elegans* was measured. The results showed that polygodial inhibited mitochondrial ATP synthesis in a dose-dependent manner (Fig. 4). The IC₅₀ for the inhibition of mitochondrial ATP synthesis was 1.8 ± 1.0 μ M. Additionally, the effect of ALA on mitochondrial ATP synthesis was measured. ALA was not able to inhibit ATP production (data not shown) even at a high concentration (100 μ M).

3.6. Morphological effect of polygodial treatment

As polygodial severely inhibits mitochondrial ATP synthesis, we studied how it affects mitochondrial morphology. A reporter strain in which a fluorescent probe is targeted to the mitochondria of all tissues was used to investigate the mitochondrial morphology. In control worms, mitochondria showed a normal network-like organization (Castelein et al., 2014), whereas the polygodial-treated worms displayed highly fragmented vesicle-like mitochondria (Fig. 5). All polygodial concentrations tested showed similar effects on the mitochondrial architecture.

SEM images (Fig. 6) showed that the cuticle of the polygodial-treated worms (100 μ M, 16 h) seemed to be released from the body. On the surface of the cuticle, the normal circumferential

annuli and furrows (Fig. 6C) disappeared after polygodial treatment and were replaced by longitudinal striations (ridges) (arrow in Fig. 6D).

4. Discussion

Medicinal plants are recognized as a rich source of bioactive compounds with therapeutic potential (Harvey et al., 2015). Searching for new anthelmintics derived from such natural sources could be a promising alternative to fight the resistance to current anthelmintic drugs (Pina-Vazquez et al., 2017). Plant-derived anthelmintic compounds have been used as leads to develop semi-synthetic or synthetic drugs with better efficacy and safety (Tagboto and Townson, 2001; Wang et al., 2010). Thus, six Kenyan medicinal plants were selected and their anthelmintic activity was tested in a *C. elegans* model. In our initial test, the ethanol and acetone extract of leaves of *W. ugandensis* showed potent activity against worms. Although *W. ugandensis* has been reported to be traditionally used for the treatment of helminthiasis and ectoparasitism in humans and animals (Nanyingi et al., 2008; Maroyi, 2014), the compounds responsible for this anthelmintic activity were still unknown. Therefore, a *W. ugandensis* ethanol extract was subjected to a bioassay-guided isolation of active compounds in this study.

Although *C. elegans* is a free-living nematode, its similarity to parasitic species suggests that it can be used as an approximate model of parasitic worms (Buckingham et al., 2014). The motility of *C. elegans* is a useful index of the effects of anthelmintic drugs. Additionally, the automated measurement of movement of worms in liquid media is well-suited for the readily scorable, phenotypic readout required for high-throughput screening (Bull et al., 2007; Buckingham and Sattelle, 2009; Buckingham et al., 2014). In our study, a WMicrotracker instrument was used to monitor the movement of worms continually. The quantitative data collected can be easily used for the potency evaluation of active compounds. However, *C. elegans* differs from mammalian

439 parasitic helminths, and further studies in e.g. a rodent helminthic model will be required in
440 anthelmintic drug discovery.

441 The bioassay-guided purification yielded three active compounds: warburganal, ALA, and
442 polygodial. These three compounds belong to two structural classes: ALA is a fatty acid, while
443 warburganal and polygodial are drimane sesquiterpenes. Although these were the three main active
444 compounds we identified, it is possible that there are other active compounds with good
445 anthelmintic activity present at lower concentrations in our extract but undetectable by our tests.
446 Nonetheless, the anthelmintic activity of our *W. ugandensis* ethanol extract can be almost
447 completely accounted for by the combined activity of the three isolated compounds.

448 ALA is an 18-carbon polyunsaturated omega-3 fatty acid with three double bonds. It has
449 been administered to humans as a food supplement for the treatment of various disorders (Cunnane
450 et al., 1993; Rallidis et al., 2003). ALA has been reported to have anthelmintic activity towards *C.*
451 *elegans*, as well as a fatty acid mixture containing linoleic acid, oleic acid, and palmitic acid as its
452 main components (Stadler et al., 1994). Another study suggests that a plant fraction containing
453 mostly medium chain fatty acids and phenolic acids has anthelmintic activity on *C. elegans* and
454 *Haemonchus contortus* (Kumarasingha et al., 2016). In addition, it was reported that medium chain
455 fatty acids have a larvicidal activity against *Toxocara cani* and *Heterobothrium okamotoi* in an in
456 vitro assay (Kiuchi et al., 1987; Hirazawa et al., 2001). These studies suggest that medium chain
457 fatty acids have anthelmintic activity. Although ALA is not potent, high doses (up to 15 g/day) can
458 be safely administered to humans or animals (Kim et al., 2014; Zehr and Walker, 2017). It has been
459 suggested that the nematicidal activity of fatty acids is due to adverse interference with the
460 nematode cuticle or hypodermis via a detergent (solubilization) effect, or through direct interaction
461 of the fatty acids with the lipophilic regions of target plasma membranes (Davis et al., 1997;
462 Williams, D.J., Kloek, A.P., Hresko, M.C., Shortt, B.J., Davila-Aponte, J.A., Bradley, J.D.,
463 McCarter, J.P., McLaird, M.B., 2003. Nematicidal fatty acid and fatty acid ester related compounds.

464 Divergence, Inc., US. WO 2003075656 A2.
465 <https://www.google.ch/patents/WO2003075656A2?cl=en>).

466 Drimane sesquiterpenes are abundant in *Warburgia* spp. Bioassay-guided purification
467 yielded in our case only warburganal and polygodial, and no other drimane sesquiterpenes. This
468 may be due to the fact that other drimane sesquiterpenes have negligible anthelmintic activity or are
469 present at low concentrations in our plant extract. Moreover, polygodial has shown activity in a
470 larval motility assay for the sheep nematode *Trichostrongylus colubriformis* in vitro (Lorimer et al.,
471 1996). To the best of our knowledge, this is the first report of the anthelmintic activity of
472 warburganal.

473 The cytotoxicity of warburganal and polygodial was significant in our study. However,
474 *Warburgia* spp. and other medicinal plants (*Polygonum hydropiper* in Asia, *Drimys* spp. in South-
475 America, *Pseudowintera* spp. in New Zealand) rich in warburganal or polygodial have been used
476 traditionally in the aforementioned regions without apparent ill effects (Wayman et al., 2010; Huq
477 et al., 2014; Barrosa et al., 2016). Perhaps the toxicity of warburganal and polygodial is mitigated
478 by other phytochemicals or the toxicity of these two compounds may be limited in vivo. Although
479 the cytotoxicity of warburganal and polygodial should be taken into account when developing this
480 class of compounds further, they could be a good starting point for the development of more potent
481 and less toxic analogues as drug candidates.

482 It is often assumed that synergy of ingredients in traditional medicinal plants will improve
483 therapeutic efficacy (Ulrich-Merzenich et al., 2010). The synergistic anthelmintic effect of fatty
484 acids and drimane sesquiterpenes has not yet been studied, and could be due to their distinct
485 mechanisms of action. Indeed, in our mitochondrial ATP synthesis measurement, ALA was not able
486 to inhibit ATP production (data not shown) even at a high concentration (100 μ M), while
487 polygodial could inhibit ATP production in a dose-dependent manner (Fig. 4). A combination of
488 ALA and polygodial or warburganal could be attractive for treating helminths by lowering the
489 possible toxicity of polygodial (warburganal), and reducing the risk of drug resistance.

Polygodial is the most potent anthelmintic compound that we isolated from *W. ugandensis*.

In addition to its anthelmintic activity, polygodial has shown a variety of pharmacological activities such as antibacterial, antifungal, analgesic, anti-inflammatory, anti-leishmanial and anti-trypanosomal effects (da Cunha et al., 2001; Andre et al., 2004; Castelli et al., 2005; Correa et al., 2011). Some of the pharmacological effects such as antinociception, anti-inflammatory and anti-allergic actions have been confirmed in in vivo animal models (Mendes et al., 2000; da Cunha et al., 2001). A phytocompound K-712, in which polygodial accounts for 30% (w:w), has been tested in a clinical trial and showed promising antifungal activity (Marotta et al., 2010; Kumari et al., 2011; Chopra et al., 2013). Further, polygodial was reported not to be mutagenic in three variants of the Ames *Salmonella* assay and using a mammal-based V79/HGPRT method (Morales et al., 1992). Taken together, this evidence suggests that polygodial is a promising lead compound for further development as an anthelmintic treatment.

Therefore, we investigated the structure-activity relationship of polygodial to further elucidate the active pharmacophore. The results indicate that the α,β -unsaturated 1,4-dialdehyde structural motif is required for potent anthelmintic activity, which can be helpful in discovering more potent and less toxic analogues (Table 4). Interestingly, in other structure-activity relationship studies of polygodial focusing on its cytotoxic, anti-fouling, or TRPA1-activating effects, the α,β -unsaturated 1,4-dialdehyde also proved essential for potent activity. It was also suggested that this dialdehyde group could react covalently with lysine residues of target proteins (Montenegro et al., 2014; Dasari et al., 2015; Mathie et al., 2017; Moodie et al., 2017).

It is important to understand the mechanism of action of anthelmintics for further target-based drug design and for the selection of effective agents to combat drug resistance (Martin, 1997). To establish the mechanism of action of polygodial, we first tested it on a panel of drug-resistant *C. elegans* mutant strains. No mutant strain was found to be significantly resistant to polygodial. This suggests that polygodial may block *C. elegans* motility through a novel mechanism, which differs from that of marketed drugs. Polygodial has been reported to activate mammalian TRP channels

(Escalera et al., 2008; Iwasaki et al., 2009; Mathie et al., 2017). Furthermore, TRP channels were proposed to be a possible anthelmintic target (Greenberg, 2014; Bais et al., 2015; Bais and Greenberg, 2016). But only one *C. elegans* strain with a TRP-channel mutant (VC602, with a *trp-2* deletion) showed slight resistance compared with wild type N2 worms. Thus it seems unlikely that polygodial would exert its anthelmintic effects via TRP-channels. Polygodial has also been reported to inhibit mitochondrial ATP synthesis in yeast and mammals (Lunde and Kubo, 2000; Castelli et al., 2005). In addition, mitochondria have also been shown to be the target of several anthelmintic hits (Kumarasingha et al., 2014; Burns et al., 2015). Our results suggest that polygodial is able to inhibit mitochondrial ATP synthesis of *C. elegans* significantly. Normally an active compound is more potent on the specific target than on the whole organism. The IC₅₀ for the inhibition of mitochondrial ATP synthesis is ~2 µM in isolated mitochondria, which is more than six times lower than that measured on whole worms. In addition, the mitochondria of polygodial-treated worms were highly fragmented (Fig. 5), and it has been previously shown that low ATP levels trigger fragmentation of the mitochondrial network (Twig and Shirihai, 2011). It is therefore likely that the polygodial-induced ATP depletion is responsible for the observed mitochondrial fragmentation. Therefore, we believe that the underlying mechanism of action of polygodial is probably inhibition of mitochondrial ATP synthesis. However, given that polygodial possesses such a diverse range of biological effects, it may well act on multiple molecular targets. It is thus possible that polygodial exerts its anthelmintic action via additional mechanisms than inhibiting mitochondrial ATP synthesis.

In conclusion, the anthelmintic activity of *W. ugandensis* is mainly ascribable to warburganal, polygodial and ALA. Polygodial (warburganal) and ALA have synergistic effects against *C. elegans*. The underlying mechanism of anthelmintic action of polygodial is probably inhibition of mitochondrial ATP synthesis. Polygodial could be a promising anthelmintic drug candidate worth further investigation.

Acknowledgement

The authors declare no competing interest. This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors. ML was supported by a Chinese Scholarship Council fellowship, and PK by an Interfaculty Council for Development Cooperation (IRO) fellowship from the KU Leuven, Belgium; WL largely supported himself. We thank Mr. Luc Vanden Bosch and Dr. Luc Van Puyvelde for help with chromatography, Dr. Lise Peeters for developing the anthelmintic assay, Ms. Caroline Vlaeminck for measuring mitochondrial ATP production, Dr. Geert Depuydt for advice on *C. elegans*, Dr. Johannes Vrijdag for the semisynthesis of drimendiol from polygodial and for NMR measurements, Prof. Josphat Matasyoh and Prof Samuel T. Kariuki Kabuitu for facilitating the collection and identification of the plants, and Prof. Johan Billen, Mr. Pieter Van de Walle and Dr. Alexander Vrijdaghs (Laboratory of Plant Conservation and Population Biology at the KU Leuven) for the SEM imaging. MS was made possible by the support of the Hercules Foundation of the Flemish Government (grant 20100225–7). Some *C. elegans* strains were obtained from the *Caenorhabditis* Genetics Center (CGC), which is funded by US National Institutes of Health Office of Research Infrastructure Programs (P40 OD010440).

560 **References**

- 561 Andre, E., Ferreira, J., Malheiros, Â., Yunes, R.A., Calixto, J.B., 2004. Evidence for the
 562 involvement of vanilloid receptor in the antinociception produced by the dialdehydes
 563 unsaturated sesquiterpenes polygodial and drimaniol in rats. *Neuropharmacology* 46, 590-
 564 597.
- 565 Atterwill, C.K., Steele, C.E., 1987. *In vitro* methods in toxicology. Cambridge University Press,
 566 UK.
- 567 Bachaya, H.A., Iqbal, Z., Khan, M.N., Sindhu, Z.U., Jabbar, A., 2009. Anthelmintic activity of
 568 *Ziziphus nummularia* (bark) and *Acacia nilotica* (fruit) against *Trichostrongylid* nematodes
 569 of sheep. *J Ethnopharmacol* 123, 325-329.
- 570 Bais, S., Churgin, M.A., Fang-Yen, C., Greenberg, R.M., 2015. Evidence for novel
 571 pharmacological sensitivities of transient receptor potential (TRP) channels in *Schistosoma*
 572 *mansoni*. *PLoS Negl Trop Dis* 9, e0004295.
- 573 Bais, S., Greenberg, R.M., 2016. TRP channels in schistosomes. *Int J Parasitol Drugs Drug Resist*
 574 6, 335-342.
- 575 Bajin Ba Ndob, I., Mengome, L.E., Bourobou Bourobou, H.P., Lossangoye Banfora, Y., Bivigou,
 576 F., 2016. Ethnobotanical survey of medicinal plants used as anthelmintic remedies in Gabon.
 577 *J Ethnopharmacol* 191, 360-371.
- 578 Barrosa, K.H., Mecchi, M.C., Rando, D.G., Ferreira, A.J., Sartorelli, P., Valle, M.M., Bordin, S.,
 579 Caperuto, L.C., Lago, J.H., Lellis-Santos, C., 2016. Polygodial, a sesquiterpene isolated
 580 from *Drimys brasiliensis* (Winteraceae), triggers glucocorticoid-like effects on pancreatic
 581 beta-cells. *Chem Biol Interact* 258, 245-256.
- 582 Brindley, P.J., Loukas, A., 2017. Helminth infection-induced malignancy. *PLoS Pathog* 13,
 583 e1006393.
- 584 Brys, K., Castelein, N., Matthijssens, F., Vanfleteren, J.R., Braeckman, B.P., 2010. Disruption of
 585 insulin signalling preserves bioenergetic competence of mitochondria in ageing
 586 *Caenorhabditis elegans*. *BMC Biol* 8, 91.
- 587 Buckingham, S.D., Partridge, F.A., Sattelle, D.B., 2014. Automated, high-throughput, motility
 588 analysis in *Caenorhabditis elegans* and parasitic nematodes: Applications in the search for
 589 new anthelmintics. *Int J Parasitol Drugs Drug Resist* 4, 226-232.
- 590 Buckingham, S.D., Sattelle, D.B., 2009. Fast, automated measurement of nematode swimming
 591 (thrashing) without morphometry. *BMC Neurosci* 10, 84.

- 592 Bull, K., Cook, A., Hopper, N.A., Harder, A., Holden-Dye, L., Walker, R.J., 2007. Effects of the
593 novel anthelmintic emodepside on the locomotion, egg-laying behaviour and development
594 of *Caenorhabditis elegans*. Int J Parasitol 37, 627-636.
- 595 Burns, A.R., Luciani, G.M., Musso, G., Bagg, R., Yeo, M., Zhang, Y., Rajendran, L., Glavin, J.,
596 Hunter, R., Redman, E., Stasiuk, S., Schertzberg, M., Angus McQuibban, G., Caffrey, C.R.,
597 Cutler, S.R., Tyers, M., Giaever, G., Nislow, C., Fraser, A.G., MacRae, C.A., Gilleard, J.,
598 Roy, P.J., 2015. *Caenorhabditis elegans* is a useful model for anthelmintic discovery. Nat
599 Commun 6, 7485.
- 600 Castelein, N., Muschol, M., Dhondt, I., Cai, H., De Vos, W.H., Dencher, N.A., Braeckman, B.P.,
601 2014. Mitochondrial efficiency is increased in axenically cultured *Caenorhabditis elegans*.
602 Exp Gerontol 56, 26-36.
- 603 Castelli, M.V., Lodeyro, A.F., Malheiros, A., Zacchino, S.A., Roveri, O.A., 2005. Inhibition of the
604 mitochondrial ATP synthesis by polygodial, a naturally occurring dialdehyde unsaturated
605 sesquiterpene. Biochem Pharmacol 70, 82-89.
- 606 Chopra, V., Marotta, F., Kumari, A., Bishier, M.P., He, F., Zerbinati, N., Agarwal, C., Naito, Y.,
607 Tomella, C., Sharma, A., Solimene, U., 2013. Prophylactic strategies in recurrent
608 vulvovaginal candidiasis: a 2-year study testing a phytonutrient vs itraconazole. J Biol Regul
609 Homeost Agents 27, 875-882.
- 610 Correa, D.S., Tempone, A.G., Reimao, J.Q., Taniwaki, N.N., Romoff, P., Favero, O.A., Sartorelli,
611 P., Mecchi, M.C., Lago, J.H., 2011. Anti-leishmanial and anti-trypanosomal potential of
612 polygodial isolated from stem barks of *Drimys brasiliensis* Miers (Winteraceae). Parasitol
613 Res 109, 231-236.
- 614 Cunnane, S.C., Ganguli, S., Menard, C., Liede, A.C., Hamadeh, M.J., Chen, Z.Y., Wolever, T.M.S.,
615 Jenkins, D.J.A., 1993. High α -linolenic acid flaxseed (*Linum usitatissimum*): some
616 nutritional properties in humans. Br J Nutr. 69, 443-453.
- 617 da Cunha, F.M., Fröde, T.S., Mendes, G.L., Malheiros, A., Cechinel Filho, V., Yunes, R.A.,
618 Calixto, J.B., 2001. Additional evidence for the anti-inflammatory and anti-allergic
619 properties of the sesquiterpene polygodial. Life Sci 70, 159-169.
- 620 Dasari, R., De Carvalho, A., Medellin, D.C., Middleton, K.N., Hague, F., Volmar, M.N., Frolova,
621 L.V., Rossato, M.F., De La Chapa, J.J., Dybdal-Hargreaves, N.F., Pillai, A., Mathieu, V.,
622 Rogelj, S., Gonzales, C.B., Calixto, J.B., Evidente, A., Gautier, M., Munirathinam, G.,
623 Glass, R., Burth, P., Pelly, S.C., van Otterlo, W.A., Kiss, R., Kornienko, A., 2015. Synthetic
624 and Biological Studies of Sesquiterpene Polygodial: Activity of 9-Epipolygodial against
625 Drug-Resistant Cancer Cells. ChemMedChem 10, 2014-2026.

- 626 Davis, E.L., Meyers, D.M., Dullum, C.J., Feitelson, J.S., 1997. Nematicidal Activity of Fatty Acid
627 Esters on Soybean Cyst and Root-knot Nematodes. *J Nematol* 29, 677-684.
- 628 Dirks-Mulder, A., Butot, R., van Schaik, P., Wijnands, J.W., van den Berg, R., Krol, L., Doebar, S.,
629 van Kooperen, K., de Boer, H., Kramer, E.M., Smets, E.F., Vos, R.A., Vrijdaghs, A.,
630 Gravendeel, B., 2017. Exploring the evolutionary origin of floral organs of *Erycina pusilla*,
631 an emerging orchid model system. *BMC Evol Biol* 17, 89.
- 632 Edwards, J., Brown, M., Peak, E., Bartholomew, B., Nash, R.J., Hoffmann, K.F., 2015. The
633 diterpenoid 7-keto-semperviol, derived from *Lycium chinense*, displays anthelmintic
634 activity against both *Schistosoma mansoni* and *Fasciola hepatica*. *PLoS Negl Trop Dis* 9,
635 e0003604.
- 636 Epe, C., Kaminsky, R., 2013. New advancement in anthelmintic drugs in veterinary medicine.
637 *Trends Parasitol* 29, 129-134.
- 638 Escalera, J., von Hehn, C.A., Bessac, B.F., Sivula, M., Jordt, S.E., 2008. TRPA1 mediates the
639 noxious effects of natural sesquiterpene deterrents. *J Biol Chem* 283, 24136-24144.
- 640 Gerlier, D., Thomasset, N., 1986. Use of MTT colorimetric assay to measure cell activation. *J*
641 *Immunol Methods* 94, 57-63.
- 642 Geurden, T., Chartier, C., Fanke, J., di Regalbono, A.F., Traversa, D., von Samson-Himmelstjerna,
643 G., Demeler, J., Vanimisetti, H.B., Bartram, D.J., Denwood, M.J., 2015. Anthelmintic
644 resistance to ivermectin and moxidectin in gastrointestinal nematodes of cattle in Europe. *Int*
645 *J Parasitol Drugs Drug Resist* 5, 163-171.
- 646 Greenberg, R.M., 2014. Ion channels and drug transporters as targets for anthelmintics. *Curr Clin*
647 *Microbiol Rep* 1, 51-60.
- 648 Gunstone, F., Pollard, M., Scrimgeour, C., Vedanayagam, H., 1977. Fatty acids. Part 50. ¹³C
649 nuclear magnetic resonance studies of olefinic fatty acids and esters. *Chem Phys Lipids* 18,
650 115-129.
- 651 Harvey, A.L., Edrada-Ebel, R., Quinn, R.J., 2015. The re-emergence of natural products for drug
652 discovery in the genomics era. *Nat Rev Drug Discov* 14, 111-129.
- 653 Hirazawa, N., Oshima, S.-i., Mitsuboshi, T., Hata, K., 2001. The anthelmintic effect of medium-
654 chain fatty acids against the monogenean *Heterobothrium okamotoi* in the tiger puffer
655 *Takifugurubripes*: evaluation of doses of caprylic acid at different water temperatures.
656 *Aquaculture* 195, 211-223.
- 657 Holden-Dye, L., Walker, R.J., 2014. Anthelmintic drugs and nematicides: studies in *Caenorhabditis*
658 *elegans*. *WormBook: the online review of C. elegans biology*, 1-29.
659 <https://www.ncbi.nlm.nih.gov/books/NBK116072/>

- 660 Huq, A.K., Jamal, J.A., Stanslas, J., 2014. Ethnobotanical, Phytochemical, Pharmacological, and
661 Toxicological Aspects of *Persicaria hydropiper* (L.) Delarbre. Evid Based Complement
662 Alternat Med 2014, 782830.
- 663 Iwasaki, Y., Tanabe, M., Kayama, Y., Abe, M., Kashio, M., Koizumi, K., Okumura, Y., Morimitsu,
664 Y., Tominaga, M., Ozawa, Y., Watanabe, T., 2009. Miogadial and miogatrial with
665 alpha,beta-unsaturated 1,4-dialdehyde moieties-Novel and potent TRPA1 agonists. Life Sci
666 85, 60-69.
- 667 Just, J., Jordan, T.B., Paull, B., Bissember, A.C., Smith, J.A., 2015. Practical isolation of polygodial
668 from *Tasmannia lanceolata*: a viable scaffold for synthesis. Org Biomol Chem 13, 11200-
669 11207.
- 670 Kaewintajuk, K., Cho, P.Y., Kim, S.Y., Lee, E.S., Lee, H.-K., Choi, E.B., Park, H., 2010.
671 Anthelmintic activity of KSI-4088 against *Caenorhabditis elegans*. Parasitol Res 107, 27-
672 30.
- 673 Katiki, L.M., Ferreira, J.F., Zajac, A.M., Masler, C., Lindsay, D.S., Chagas, A.C.S., Amarante,
674 A.F., 2011. *Caenorhabditis elegans* as a model to screen plant extracts and compounds as
675 natural anthelmintics for veterinary use. Vet Parasitol 182, 264-268.
- 676 Kayser, E.-B., Morgan, P.G., Hoppel, C.L., Sedensky, M.M., 2001. Mitochondrial expression and
677 function of GAS-1 in *Caenorhabditis elegans*. J Biol Chem 276, 20551-20558.
- 678 Kim, K.B., Nam, Y.A., Kim, H.S., Hayes, A.W., Lee, B.M., 2014. alpha-Linolenic acid:
679 nutraceutical, pharmacological and toxicological evaluation. Food Chem Toxicol 70, 163-
680 178.
- 681 Kiuchi, F., Miyashita, N., Tsuda, Y., Kondo, K., Yoshimura, H., 1987. Studies on crude drugs
682 effective on visceral larva migrans. I. Identification of larvicidal principles in betel nuts.
683 Chem Pharm Bull (Tokyo) 35, 2880-2886.
- 684 Kumarasingha, R., Karpe, A.V., Preston, S., Yeo, T.C., Lim, D.S., Tu, C.L., Luu, J., Simpson, K.J.,
685 Shaw, J.M., Gasser, R.B., Beale, D.J., Morrison, P.D., Palombo, E.A., Boag, P.R., 2016.
686 Metabolic profiling and *in vitro* assessment of anthelmintic fractions of *Picria fel-terrae*
687 *Lour.* Int J Parasitol Drugs Drug Resist 6, 171-178.
- 688 Kumarasingha, R., Palombo, E., Bhawe, M., Yeo, T., Lim, D., Tu, C., Shaw, J., Boag, P., 2014.
689 Enhancing a search for traditional medicinal plants with anthelmintic action by using wild
690 type and stress reporter *Caenorhabditis elegans* strains as screening tools. Int J Parasitol 44,
691 291-298.
- 692 Kumari, A., Bishier, M.P., Naito, Y., Sharma, A., Solimene, U., Jain, S., Yadava, H., Minelli, E.,
693 Tomella, C., Marotta, F., 2011. Protective effect of an oral natural phytonutrient in recurrent
694 vulvovaginal candidiasis: a 12-month study. J Biol Regul Homeost Agents 25, 543-551.

- 695 Laing, R., Maitland, K., Lecova, L., Skuce, P.J., Tait, A., Devaney, E., 2016. Analysis of putative
696 resistance gene loci in UK field populations of *Haemonchus contortus* after 6 years of
697 macrocyclic lactone use. *Int J Parasitol* 46, 621-630.
- 698 Lee, S.O., Choi, S.Z., Choi, S.U., Ryu, S.Y., Lee, K.R., 2004. Phytochemical constituents of the
699 aerial parts from *Aster hispidus*. *Nat Prod Sci* 10, 335-340.
- 700 Leonard, C.M., Viljoen, A.M., 2015. *Warburgia*: a comprehensive review of the botany, traditional
701 uses and phytochemistry. *J Ethnopharmacol* 165, 260-285.
- 702 Li, R.W., Rinaldi, M., Capuco, A.V., 2011. Characterization of the abomasal transcriptome for
703 mechanisms of resistance to gastrointestinal nematodes in cattle. *Vet Res* 42, 114.
- 704 Lorimer, S.D., Perry, N.B., Foster, L.M., Burgess, E.J., Douch, P.G., Hamilton, M.C., Donaghy,
705 M.J., McGregor, R.A., 1996. A nematode larval motility inhibition assay for screening plant
706 extracts and natural products. *J Agric Food Chem* 44, 2842-2845.
- 707 Loukas, A., Hotez, P.J., Diemert, D., Yazdanbakhsh, M., McCarthy, J.S., Correa-Oliveira, R.,
708 Croese, J., Bethony, J.M., 2016. Hookworm infection. *Nat Rev Dis Primers* 2, 16088.
- 709 Lunde, C.S., Kubo, I., 2000. Effect of polygodial on the mitochondrial ATPase of *Saccharomyces*
710 *cerevisiae*. *Antimicrob Agents Chemother* 44, 1943-1953.
- 711 Marotta, F., Naito, Y., Bishier, M.P., Jain, S., Yadav, H., Minelli, E., Kumari, A., Solimene, U.,
712 Sollano, J., 2010. Subclinical candiduria in patients with gastrointestinal malignancies: a
713 preliminary study on the protective effect of a natural phytocompound. *J Biol Regul*
714 *Homeost Agents* 24, 317-324.
- 715 Maroyi, A., 2014. The genus *Warburgia*: a review of its traditional uses and pharmacology. *Pharm*
716 *Biol* 52, 378-391.
- 717 Martin, R.J., 1997. Modes of action of anthelmintic drugs. *Vet J* 154, 11-34.
- 718 Mathie, K., Lainer, J., Spreng, S., Dawid, C., Andersson, D.A., Bevan, S., Hofmann, T., 2017.
719 Structure-Pungency Relationships and TRP Channel Activation of Drimane Sesquiterpenes
720 in Tasmanian Pepper (*Tasmannia lanceolata*). *J Agric Food Chem* 65, 5700-5712.
- 721 Mendes, G.L., Santos, A.R.S., Malheiros, A., Cechinel, V., Yunes, R.A., Calixto, A.B., 2000.
722 Assessment of mechanisms involved in antinociception caused by sesquiterpene polygodial.
723 *J Pharmacol Exp Ther* 292, 164-172.
- 724 Montenegro, I., Tomasoni, G., Bosio, C., Quinones, N., Madrid, A., Carrasco, H., Olea, A.,
725 Martinez, R., Cuellar, M., Villena, J., 2014. Study on the cytotoxic activity of drimane
726 sesquiterpenes and nordrimane compounds against cancer cell lines. *Molecules* 19, 18993-
727 19006.
- 728 Moodie, L.W.K., Trepos, R., Cervin, G., Larsen, L., Larsen, D.S., Pavia, H., Hellio, C., Cahill, P.,
729 Svenson, J., 2017. Probing the Structure-Activity Relationship of the Natural Antifouling

- 730 Agent Polygodial against both Micro- and Macrofoulers by Semisynthetic Modification. J
731 Nat Prod 80, 515-525.
- 732 Morales, P., Andersson, M., Lewan, L., Sterner, O., 1992. Structure-activity relationships for
733 unsaturated dialdehydes. 6. The mutagenic activity of 11 compounds in the V79/HGPRT
734 assay. Mutat Res 268, 315-321.
- 735 Nanyingi, M.O., Mbaria, J.M., Lanyasunya, A.L., Wagate, C.G., Koros, K.B., Kaburia, H.F.,
736 Munenge, R.W., Ogara, W.O., 2008. Ethnopharmacological survey of Samburu district,
737 Kenya. J Ethnobiol Ethnomed 4, 14.
- 738 Norhayati, M., Fatmah, M., Yusof, S., Edariah, A., 2003. Intestinal parasitic infections in man: a
739 review. Med J Malaysia 58, 296-305.
- 740 Odds, F.C., 2003. Synergy, antagonism, and what the chequerboard puts between them. J
741 Antimicrob Chemother 52, 1.
- 742 Orhan, G., Bayram, A., Zer, Y., Balci, I., 2005. Synergy tests by E test and checkerboard methods
743 of antimicrobial combinations against *Brucella melitensis*. J Clin Microbiol 43, 140-143.
- 744 Pina-Vazquez, D.M., Mayoral-Pena, Z., Gomez-Sanchez, M., Salazar-Olivo, L.A., Arellano-
745 Carbajal, F., 2017. Anthelmintic effect of *Psidium guajava* and *Tagetes erecta* on wild-type
746 and Levamisole-resistant *Caenorhabditis elegans* strains. J Ethnopharmacol 202, 92-96.
- 747 Porta-de-la-Riva, M., Fontrodona, L., Villanueva, A., Ceron, J., 2012. Basic *Caenorhabditis*
748 *elegans* methods: synchronization and observation. J Vis Exp 64, e4019.
- 749 Rallidis, L.S., Paschos, G., Liakos, G.K., Velissaridou, A.H., Anastasiadis, G., Zampelas, A., 2003.
750 Dietary alpha-linolenic acid decreases C-reactive protein, serum amyloid A and interleukin-
751 6 in dyslipidaemic patients. Atherosclerosis 167, 237-242.
- 752 Rana, A.K., Misra-Bhattacharya, S., 2013. Current drug targets for helminthic diseases. Parasitol
753 Res 112, 1819-1831.
- 754 Romero-Benavides, J.C., Ruano, A.L., Rivas, R.S., Veintimilla, P.C., Jaramillo, S.V., Bailon-
755 Moscoso, N., 2017. Medicinal plants used as anthelmintics: Ethnomedical, pharmacological,
756 and phytochemical studies. Eur J Med Chem 129, 209-217.
- 757 Salas, A.L., Alberto, M.R., Zampini, I.C., Cuello, A.S., Maldonado, L., Rios, J.L., Schmeda-
758 Hirschmann, G., Isla, M.I., 2016. Biological activities of polyphenols-enriched propolis
759 from Argentina arid regions. Phytomedicine 23, 27-31.
- 760 Satou, T., Kaneko, K., Li, W., Koike, K., 2008. The toxin produced by *Pleurotus ostreatus* reduces
761 the head size of nematodes. Biol Pharm Bull 31, 574-576.
- 762 Stadler, M., Mayer, A., Anke, H., Sterner, O., 1994. Fatty acids and other compounds with
763 nematicidal activity from cultures of Basidiomycetes. Planta Med 60, 128-132.

- 764 Tagboto, S., Townson, S., 2001. Antiparasitic properties of medicinal plants and other naturally
765 occurring products. *Adv Parasitol* 50, 199-295.
- 766 Taman, A., Azab, M., 2014. Present-day anthelmintics and perspectives on future new targets.
767 *Parasitol Res* 113, 2425-2433.
- 768 Twig, G., Shirihai, O.S., 2011. The interplay between mitochondrial dynamics and mitophagy.
769 *Antioxid Redox Signal* 14, 1939-1951.
- 770 Ulrich-Merzenich, G., Panek, D., Zeitler, H., Vetter, H., Wagner, H., 2010. Drug development from
771 natural products: exploiting synergistic effects. *Indian J Exp Biol* 48, 208-219.
- 772 Urones, J.G., Marcos, I.S., Pérez, B.G., Díez, D., Lithgow, A.M., Gómez, P.M., Basabe, P.,
773 Garrido, N.M., 1994. Chemistry of zamoranic acid. Part V Homochiral semisyntheses of
774 active drimanes: Pereniporin B, polygodial and warburganal. *Tetrahedron* 50, 10995-11012.
- 775 Walker, J.M., 1994. The bicinchoninic acid (BCA) assay for protein quantitation. *Methods Mol*
776 *Biol* 32, 5-8.
- 777 Wang, G.X., Han, J., Zhao, L.W., Jiang, D.X., Liu, Y.T., Liu, X.L., 2010. Anthelmintic activity of
778 steroidal saponins from *Paris polyphylla*. *Phytomedicine* 17, 1102-1105.
- 779 Wangchuk, P., Giacomini, P.R., Pearson, M.S., Smout, M.J., Loukas, A., 2016a. Identification of
780 lead chemotherapeutic agents from medicinal plants against blood flukes and whipworms.
781 *Sci Rep* 6, 32101.
- 782 Wangchuk, P., Pearson, M.S., Giacomini, P.R., Becker, L., Sotillo, J., Pickering, D., Smout, M.J.,
783 Loukas, A., 2016b. Compounds Derived from the Bhutanese Daisy, *Ajania nubigena*,
784 Demonstrate Dual Anthelmintic Activity against *Schistosoma mansoni* and *Trichuris muris*.
785 *PLoS Negl Trop Dis* 10, e0004908.
- 786 Wayman, K.A., de Lange, P.J., Larsen, L., Sansom, C.E., Perry, N.B., 2010. Chemotaxonomy of
787 *Pseudowintera*: sesquiterpene dialdehyde variants are species markers. *Phytochemistry* 71,
788 766-772.
- 789 Weaver, K.J., May, C.J., Ellis, B.L., 2017. Using a health-rating system to evaluate the usefulness
790 of *Caenorhabditis elegans* as a model for anthelmintic study. *PloS one* 12, e0179376.
- 791 Zehr, K.R., Walker, M.K., 2018. Omega-3 polyunsaturated fatty acids improve endothelial function
792 in humans at risk for atherosclerosis: A review. *Prostaglandins Other Lipid Mediat* 134, 131-
793 140.
- 794

Figure legends

Fig. 1. Bioassay-guided purification of active anthelmintic compounds. (A) Anthelmintic activity (mean $\pm$ S.D., $n = 2$) of small-scale extract (200 μ g/mL) of six medicinal plants using four solvents. (B) Anthelmintic activity (mean $\pm$ S.D., $n = 2$) of fractions from a preparative silica gel column (100 μ g/mL) of *Warburgia ugandensis*. (C) HPLC chromatogram (203 nm) of fraction F3 resolved on a C18 column at a flow rate of 4.0 mL/min. The mobile phase was a mixture of acetonitrile and MilliQ® water, both with 0.05% trifluoroacetic acid; the gradient is indicated by a solid blue line. (D) Anthelmintic activity (mean $\pm$ S.D., $n = 2$) of HPLC fractions of F3. Statistical analysis was done using one-way ANOVA with Dunnett's test to assess the samples versus DMSO control. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$.

Fig. 2. Structures of active compounds isolated from the leaves of *Warburgia ugandensis* and the dose-response curve of their anthelmintic activity (mean $\pm$ S.D., $n = 3$). Data from dose-response experiments were represented as the percentage of inhibition, and analyzed with GraphPad Prism 6 software (San Diego, CA, USA). A log (inhibitor) versus response non-linear fit was used to estimate the IC_{50} . ALA, alpha-linolenic acid.

Fig. 3. The anthelmintic activity (EC_{50}) (mean $\pm$ S.D., $n = 3$) of polygodial on drug-resistant and transient receptor potential (TRP) mutant *Caenorhabditis elegans* strains. (A) Drug-resistant mutant strains (for details of strains, see Table 1); (B) TRP mutant strains (for details of strains, see Table 2). Data from dose-response experiments were represented as the percentage of inhibition and analyzed with GraphPad Prism 6 software (San Diego, CA, USA). A log (inhibitor) versus response non-linear fit was used to estimate the IC_{50} . Statistical analysis was done by one-way ANOVA with Dunnett's test to assess the samples versus DMSO control. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$.

Fig. 4. The effect of polygodial on mitochondrial ATP synthesis (mean $\pm$ S.D., $n = 3$) of *Caenorhabditis elegans*. Oligomycin (4 μ g/mL) was used as a positive control in this assay.

Fig. 5. The effect of polygodial on the mitochondrial morphology of young adult *Caenorhabditis elegans* was assessed using confocal images. Representative images of the mitochondrial network in the intestine of the worm after 3 h treatment with 0.5% DMSO (A), 20 μ M polygodial (B), 40 μ M polygodial (C) and 100 μ M polygodial (D) are shown. Arrows indicate mitochondrial fragmentation. Scale bar = 10 μ m. These images are representative of three worms per condition.

Fig. 6. Scanning electron micrograph of the lip region (A, B) and cuticle surface (C, D) of young adult *Caenorhabditis elegans*, after treatment for 16 h with DMSO (0.5%) (solvent control) (A, C) or polygodial (100 μ M) (B, D). Magnification: 7500 $\times$ (except for B: 3500 $\times$). Ridges in the cuticle are indicated by a white arrow. These images are representative of three worms per condition.

837 **Table 1.** Drug-resistant mutant strains of *Caenorhabditis elegans* used in the study.

838

Strain	Genotype	Known Resistances
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
839	DMPP, 1,1-dimethyl-4-phenylpiperazinium.	

840

841

842 **Table 2.** Transient receptor potential (TRP) channel mutant strains of *Caenorhabditis elegans* used in the
843 study.

844

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

845

846

Table 3. Cytotoxicity of warburganal, alpha-linolenic acid (ALA) and polygodial on HEK 293 and RAW 264.7 cell lines.^a

Compound		HEK 293	RAW 264.7
warburganal	CC ₅₀ ^b (μM)	47.7±7.9	49.6±8.4
ALA	CC ₅₀ (μM)	298.3±25.4	363.8±32.1
polygodial	CC ₅₀ (μM)	58.1±8.6	64.8±10.2

^aCell lines were purchased from ATCC (USA).

^b CC₅₀ is the calculated concentration of a compound that leads to 50% inhibition.

856 **Table 4.** The anthelmintic activity of polygodial and its analogues.

857

Structure	Name or code name	IC ₅₀ (μM)
polygodial	13.1±5.3
warburganal	28.2±8.6
mukaadial	23.7±7.4
drimenol	49.1±10.1
drimendiol	> 200
ugandenial A	> 200
Sigma1	235.1±21.4
Sigma2	> 300

Specs1

> 300

Specs2

> 300

Specs3

> 300

Specs4

> 300

Specs5

155.1±25.3

Specs6

> 300

Specs7

240.0±25.1

Specs8

> 300

Specs9

> 300

Specs10

93.7±20.4

Specs11

285.4±30.5

858

859

Highlights

- Bioassay-guided isolation of anthelmintic compounds from *Warburgia ugandensis* leaves using *Caenorhabditis elegans* model
- Anthelmintic activity of *W. ugandensis* is mainly ascribable to warburganal, polygodial and alpha-linolenic acid (ALA)
- Polygodial and ALA , as well as warburganal and ALA, have synergistic effects against *C. elegans*
- The underlying mechanism of anthelmintic action of polygodial is probably inhibition of mitochondrial ATP synthesis