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Selected α-pyrones from the plants *Cryptocarya novoguineensis* (Lauraceae) and *Piper methysticum* (Piperaceae) with activity against *Haemonchus contortus* in vitro

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ABSTRACT

Due to the widespread occurrence and spread of anthelmintic resistance, there is a need to develop new drugs against resistant parasitic nematodes of livestock animals. The Nobel Prize-winning discovery and development of the anti-parasitic drugs avermectin and artemisinin has renewed the interest in exploring natural products as anthelmintics. In the present study, we screened 7500 plant extracts for in vitro-activity against the barber's pole worm, *Haemonchus contortus*, a highly significant pathogen of ruminants. The anthelmintic extracts from two plants, *Cryptocarya novoguineensis* and *Piper methysticum*, were fractionated by high-performance liquid chromatography (HPLC). Subsequently, compounds were purified from fractions with significant biological activity. Four α-pyrones, namely goniothalamin (GNT), dihydrokavain (DHK), desmethoxyyangonin (DMY) and yangonin (YGN), were purified from fractions from the two plants, GNT from *C. novoguineensis*, and DHK, DMY and YGN (= kavalactones) from *P. methysticum*. The three kavalactones induced a lethal, eviscerated (Evi) phenotype in treated exsheathed third-stage larvae (xL3s), and DMY and YGN had moderate potencies (IC_{50} values of 31.7 ± 0.23 μM and 23.7 ± 2.05 μM, respectively) at inhibiting the development of xL3s to fourth-stage larvae (L4s). Although GNT had limited potency (IC_{50} of 200–300 μM) at inhibiting L4 development, it was the only compound that reduced L4 motility (IC_{50} of 6.25–12.50 μM). The compounds purified from each plant affected *H. contortus* in an irreversible manner. These findings suggest that structure-activity relationship studies of α-pyrones should be pursued to assess their potential as anthelmintics.

1. Introduction

Natural products have an enormous chemical and structural diversity, which is unmatched by synthetic libraries of small molecules (Harvey et al., 2015) and, thus, inspire new discoveries in chemistry, biology and medicine (Shen, 2015; Newman and Cragg, 2016). There are numerous examples of natural products that have substantially advanced drug discovery and chemotherapy (cf. Strobel and Daisy, 2003; Shen, 2015). Key examples include the Nobel Prize-winning discoveries of penicillin by Alexander Fleming, Ernst Chain and Howard Florey (1945) and streptomycin by Selman A. Waksman (1952), representing the start of a 'golden age' of natural product-drug discovery in the 1950s and 1960s, respectively.

In subsequent years, major advances in combinatorial chemistry (Liu et al., 2017) underpinned the large-scale synthesis of compounds (Houghten et al., 1991) and the establishment and curation of synthetic libraries of small molecules for high throughput screening (Liu et al., 2017), somewhat marginalising natural product-based drug discovery (Harvey, 2008). However, this change led to a major reduction in novel lead compounds entering the drug development pipeline and in new drugs entering the market (Li and Vederas, 2009). Nonetheless, there has been an increased interest again in drugs from natural compounds

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since the 2015 Nobel Prize in Physiology or Medicine was awarded to William Campbell, Satoshi Omura and Youyou Tu for their discoveries of avermectin or artemisinin, which led to major success in the control of human filariases and malaria (Shen, 2015; Campbell, 2016). These advances were clearly major milestones in parasitology, providing new hope and aspirations for the discovery of new antiparasitic compounds (Shen, 2015).

The discovery of new chemotherapeutics is imperative in the veterinary area, given the widespread problems associated with drug resistance, particularly in populations of parasitic roundworms (nematodes) of livestock animals (Geary, 2016; Kotze and Prichard, 2016). Even though new anthelmintics, such as monepantel (Kaminsky et al., 2008; Prichard and Geary, 2008) and derquantel (Little et al., 2010), were introduced relatively recently, resistance to these compounds have been reported (Kaminsky et al., 2011; Scott et al., 2013). Thus, in order to reduce productivity losses and diseases linked to nematode infections (Lane et al., 2015), there is an urgent and continued need to discover and develop new compounds against nematodes of livestock.

Over the last years, we have been working toward this goal, with an emphasis on the discovery of novel nematocidal or nematostatic compounds for medical chemistry optimisation and subsequent development (e.g., Preston et al., 2016, 2017a; Herath et al., 2017; Jiao et al., 2017). The Griffith Institute for Drug Discovery maintains multiple compound collections, including NatureBank which contains natural product extracts (n = 10,000) and fractions (n = 50,000) derived from plants, marine invertebrates and fungi as well as 30,000 archived biota samples linked to most of these samples (Camp et al., 2014). In the present study, we took advantage of these resources and screened thousands of plant extracts for their anti-parasitic activity against the barber's pole worm, *Haemonchus contortus*, a highly significant pathogen of ruminants, and then characterised and assessed nematocidal fractions and pure compounds derived from the plants *Cryptocarya novoguineensis* (family Lauraceae) and *Piper methysticum* (family Piperaceae).

2. Materials and methods

2.1. NatureBank extract library

The NatureBank library from the Griffith Institute for Drug Discovery contained extracts from plants (75%; 7500), marine invertebrates (20%; 2000) and fungi (5%; 500). The methods employed for the preparation of these extracts (defined as lead-like enhanced [LLE] extracts) have been described previously (Camp et al., 2011). In the present study, aliquots of 7500 plant extracts in the NatureBank library were purchased and used; the dried extracts were individually solubilised in dimethyl sulfoxide (DMSO; Ajax Finechem, Australia) to achieve a stock concentration of 250 µg equivalents per µl (µge/µl).

The concentration units, µge/µl, used for the NatureBank extract library relate to: (i) the amount of dry plant material that is weighed out for extraction, and (ii) the amount of DMSO that the dry plant extract is dissolved in to make the extract for subsequent screening. For example, 300 mg equivalents (mge) is the extract derived from 300 mg of plant material; when this extract is dissolved in 1.2 ml of solvent (i.e. DMSO), then the final stock solution concentration is 250 µge/µl. Further details on this methodology have been published previously (Camp et al., 2013).

2.2. Fractionation of 'hit' extracts from plants, and assessment of purity

First, air-dried and ground leaves (10.5 g) or roots (10.6 g) were extracted with n-hexane (250 ml, 2 h), CH_2Cl_2 (250 ml, 2 h) and MeOH (250 ml, 2 h; 250 ml, 16 h). The n-hexane extract was discarded, while the CH_2Cl_2 and MeOH extracts were combined and dried under reduced pressure to yield a dark green gum (0.90–1.61 g). This material was resuspended in MeOH (150 ml), loaded on to a MeOH-conditioned polyamide gel (Machery Nagel Polyamide CC6 (0.05–0.016 mm, 30 g) in a sintered glass column, and washed with MeOH (300 ml); the resultant MeOH flush was dried to yield a green gum (LLE extracts; 0.66–1.36 g).

A portion of this material (0.20 g) was pre-adsorbed on Alltech C_{18}-bonded silica (35–75 µm, 150 Å) and dry-packed into an Alltech stainless steel guard cartridge (10 × 30 mm), which was then attached to a Thermo Betasil C_{18}-bonded silica 5 µm column (21.2 mm × 150 mm). Isocratic high-performance liquid chromatography (HPLC) conditions of 90% H_2O (0.1% TFA)/10% MeOH (0.1% TFA) were employed for the first 10 min; then, a linear gradient to MeOH (0.1% TFA) was run for 40 min, followed by isocratic conditions of MeOH (0.1% TFA) for 10 min, all at a flow rate of 9 ml/min. A Waters 600 pump, fitted with a Waters 996 photodiode array detector and a Gilson 215 liquid handler equipped with a Gilson 819 injector, was used for semi-preparative HPLC separations. Sixty fractions were collected by time (60 × 1 min) from the start of the HPLC run and prepared for screening in the bioassays (Subsection 2.4). The MeOH (Honeywell, USA), n-hexane and CH_2Cl_2 (ACI-Labscan, Thailand) used for extractions and chromatography were all HPLC grade. TFA was purchased from Sigma-Aldrich (USA).

The fractions from plant extracts with significant biological activity against *H. contortus* (cf. Subsection 2.4) were individually analysed by ^{1}H nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS), and then purified further by HPLC using C_{18}-bonded silica as a stationary phase. The purified compounds were identified by comparing NMR and MS data with those obtained from published literature (Jewers et al., 1972; Dharmaratne et al., 2002). Specific optical rotations were acquired on a JASCO P-1020 polarimeter, and NMR spectra were recorded on a Bruker AVANCE HDX 800 MHz NMR spectrometer, equipped with a TCI cryoprobe at 25 °C. NMR spectra were processed using MestReNova v.11.0 and the ^{1}H and ^{13}C NMR chemical shifts were referenced to the solvent peaks for $CDCl_3$ at δ_H = 7.26 ppm and δ_C = 77.16 ppm, respectively. Low resolution ESI-MS data were recorded on a Thermo MSQ Plus mass spectrometer. The solvents used for specific rotation and MS were HPLC grade from B&J (USA). Subsequently, compounds that showed effects in *H. contortus* bioassays were purchased from the company PhytoLab (Germany) and confirmed to be > 95% pure by ^{1}H-NMR spectroscopy.

2.3. Parasite production and maintenance

Haemonchus contortus (Haecon-5 strain) was produced in experimental sheep as described previously (Schwarz et al., 2013; Preston et al., 2015), in accord with institutional animal ethics guidelines (permit no. 1413429; The University of Melbourne, Australia). L3s were cultured, stored and exsheathed employing established protocols (Preston et al., 2015). In brief, L3s were exsheathed by incubation in 0.15% v/v sodium hypochlorite (NaClO) for 20 min at 37 °C and then washed five times in sterile, physiological saline (pH 7.0, 37 °C). Then, xL3s were cultured in Luria Bertani (LB) medium supplemented with 100 IU/ml of penicillin, 100 mg/ml of streptomycin and 2.5 mg/ml of amphotericin (supplemented medium was called LB*; Preston et al., 2015). L4s were produced by incubating xL3s in LB* at 10% v/v CO_2 and 38 °C for 7 days (Preston et al., 2015).

2.4. Bioassay for screening of plant extracts and chromatographic fractions on H. contortus

Individual NatureBank plant extracts were screened in an established *H. contortus* bioassay (Preston et al., 2015) for a reduction in motility of xL3s; the compounds monepantel and moxidectin (20 µM) were included as positive controls, and LB* + 1.25% DMSO as a negative (untreated) control. In brief, extracts were diluted to a final concentration of 3 µge/µl in LB*, and arrayed (50 µl per well) into 96-well plates using an automated multichannel pipette (Viaflo Assist/II,

Integra Biosciences, Switzerland). Then, xL3s were dispensed (300 xL3s in 50 µl per well) into individual wells, and the plates were incubated (10% v/v CO_2, 38 °C, model 311, Thermo Fisher Scientific, USA) for 72 h. A video recording (5 s) was made at 72 h, and the motility index (Mi) value was recorded in individual wells as described previously (Preston et al., 2015). The percentage of xL3 motility reduction in each well was calculated using the program Prism (v.7.02 GraphPad Software). An extract was recorded as a 'hit' (i.e. being active) if it reduced xL3 motility by ≥70% after 72 h. Fractions from active ('hit') extracts were then tested individually for inhibition of xL3 motility and of L4 development (cf. Preston et al., 2015). To assess the inhibition of L4 development, the xL3s were treated with the fractions and the number of L4s that developed in 7 days were counted. L4s were differentiated from xL3s by their well-developed mouth and pharynx (cf. Preston et al., 2015). The results were analysed using a non-parametric one-way ANOVA and Dunnett's multiple comparison tests, as implemented in GraphPad Prism v7.02. The fractions with a significant inhibition were selected for the characterisation and purification of compounds.

2.5. Assessment of potency of extracts and compounds on motility, development and morphological phenotype of H. contortus

Individual 'hit' extracts or purified compounds were tested for their potency at inhibiting the motility of *H. contortus* xL3s or L4s, and of L4 development, in a two-fold dilution series [18 points, commencing at 3 µge/µl (+1.25% DMSO) for extracts, and 100 µM (+0.25% DMSO) for purified test and control compounds - monepantel and moxidectin]. LB* plus matched percentages of DMSO were included as negative (untreated) controls. To assess motility, xL3s or L4s were treated with the individual extracts or compounds, and 5 s video recordings were made of individual wells at 24 h, 48 h and 72 h (cf. Preston et al., 2015). The compounds were tested further for the inhibition of L4 development and motility at concentrations of 500 µM, 400 µM, 300 µM and 200 µM, with matched concentrations of monepantel and moxidectin being included as positive controls, and of DMSO (commencing at 1.25% DMSO) as negative (untreated) controls. Both the development and motility assays were repeated three times, with three technical replicates in each assay. The concentration at half-maximum response (IC_{50}) of each extract or compound was determined by transforming the concentration to $\log_{10}$ and fitting to a variable slope four-parameter model using GraphPad Software.

Subsequently, the irreversibility of the effects of individual compounds on xL3s following removal from the medium was evaluated. For this purpose, the percentages of larvae (xL3 and L4) were counted at different time points (from 24 h to 72 h at 6-h intervals and at 7 days) of compound exposure, and at 7 days following compound removal at different time points (from 24 h to 72 h at 6-h intervals) (Fig. 3). Two biological replicates (each with three technical replicates) were performed for representative compounds at concentrations that inhibited L4 development by ≥80% (7 days). The statistical analyses were conducted using one-way ANOVA (non-parametric) and Dunnett's multiple comparison tests, in order to compare of L4 development following the removal of compounds from xL3 cultures at well-defined time points (from 24 h to 72 h at 6-h intervals) to L4 development with compound present throughout the 7 days of the assay.

To study the effect of concentrations of active compounds on the morphological phenotype of *H. contortus*, they were tested in a dilution series (300 µM, 200 µM, 100 µM, 50 µM, 25 µM, 12.5 µM and 6.25 µM), and the percentages of affected xL3s were counted (at 7 days). In addition, the earliest time point of appearance of a phenotype was determined by scoring the percentage of affected xL3s at different time points (from 24 h to 72 h at 6-h intervals) of compound exposure (100 µM).

2.6. Cytotoxicity and selectivity of compounds

The toxicity of the compounds to a normal breast epithelial cell line (MCF10A) was assessed in vitro in two biological assays (duplicates in each assay) as described previously (Preston et al., 2017b). In brief, MCF10A cells in Dulbecco's Modified Eagle Medium were cultured at a density of 700 per well, and then treated with individual compounds or controls. Monepantel (activity on larvae) and doxorubicin (cell toxicity) were used as positive controls, and culture medium with matched DMSO concentration as a negative (untreated) control. Test and control compounds were tested in a 10-point two-fold dilution series, commencing at the concentrations that inhibited L4 development by ≥80%, and at 50 µM and 10 µM for monepantel and doxorubicin, respectively. After 48 h of incubation, MCF10A cells were stained with 4′,6-diamidino-2-phenylindole (DAPI; 5 µg/ml), an image of each well was taken, and the numbers of nuclei in individual wells were counted using an established, automated protocol (see Preston et al., 2017b). IC_{50} values were calculated as described in Subsection 2.5, and the selectivity index (SI) was calculated as per IC_{50} for MCF10A cells/IC_{50} for *H. contortus* (see Preston et al., 2017b).

3. Results

3.1. Identification of active extracts and chromatographic fractions

Through the screening of the 7500 extracts, we identified three plant extracts (*Cn*-L, *Cn*-R and *Pm*-R) that consistently inhibited xL3 motility by 70%; *Cn*-L was a leaf extract from *Cryptocarya novoguineensis*, *Cn*-R was a root extract from the same plant species, and *Pm*-R was an extract from the roots of *Piper methysticum* (Table 1). Although the inhibition of xL3 motility was not dose-dependent for these extracts (not shown), each of the three extracts elicited a dose-dependent inhibition of L4 development (Fig. 1; Table 1), with *Pm*-R having the highest potency in the L4 developmental assay (IC_{50} = 0.16 ± 0.06 µge/µl; see Fig. 1; Table 1).

Subsequently, the three extracts (designated *Cn*-L, *Cn*-R and *Pm*-R), each with activity against *H. contortus*, were subjected to chromatographic separation, and 60 fractions from each extract were tested in the xL3 motility and L4 developmental assays. Given the lack of dose-response effects of extracts, none of the fractions inhibited xL3 motility (not shown). Fraction 40 from each *Cn*-L and *Cn*-R elicited a significant ($P < 0.0001$) inhibition of L4 development (Fig. 1), and fractions 41–42 and 44–45 from *Pm*-R displayed the most significant ($P < 0.0001$) developmental inhibition (Fig. 1).

3.2. Chemical analyses of active fractions and identification of compounds

The NMR and MS analyses of fraction 40 of each *Cn*-L and *Cn*-R revealed pure goniothalamin (GNT; 27.4–31.3 mg, 0.86–0.98% dry weight) (Supplementary File 1; cf. Jewers et al., 1972). Separate analyses of fractions 41 and 42 of *Pm*-R revealed dihydrokavain (DHK; 6.9 mg, 0.43% dry weight) (Supplementary File 1; cf. Dharmaratne et al., 2002) in both fractions. Analyses of fractions 44 and 45 of *Pm*-R revealed desmethoxyyangonin (DMY; 2.8 mg, 0.172% dry weight) (Supplementary File 1; cf. Dharmaratne et al., 2002) and yangonin (YGN; 2.4 mg, 0.15% dry weight) (cf. Dharmaratne et al., 2002), respectively. Thus, we succeeded in purifying four known compounds (GNT, DHK, DMY and YGN; see Fig. 2), all belonging to the α-pyrone structural class (Drewes et al., 1995; Smith et al., 2004).

3.3. Potency of α-pyrones at inhibiting L4 development and L4 motility

All four α-pyrones (GNT, DHK, DMY and YGN) were individually tested for their inhibitory effects on L4 development and motility. Two analogues, DMY and YGN, inhibited L4 development with IC_{50} values of 31.7 ± 0.23 µM and 23.7 ± 2.05 µM, respectively (at 7 days), whereas GNT and DHK had higher IC_{50} values (Fig. 2; Table 1).

Of the four α-pyrones tested, GNT elicited a dose-dependent inhibition of L4 motility and induced a 'straight' phenotype in treated L4s

Table 1

Details and in vitro activity and/or cytotoxicity of three plant extracts and four purified compounds. Extracts were from leaves (*Cn*-L) or roots (*Cn*-R) of *Cryptocarya novoguineensis*, from roots (*Pm*-R) of *Piper methysticum*, and the four purified compounds were goniothalamin (GNT), dihydrokavain (DHK), desmethoxyyangonin (DMY) and yangonin (YGN). Comparison of the half-maximum inhibitory concentration (IC_{50}) values for the inhibitory effects of these extracts or compounds on L4 development and/or L4 motility of *Haemonchus contortus*. IC_{50} values are expressed as a mean IC_{50} ± standard error of mean or a range compared with respective values for monepantel (MON) and moxidectin (MOX). IC_{50} values for toxicity on MCF10A cells and selectivity indices (SI) relating L4 development or L4 motility for purified compounds were compared with those values for MON.

Details and in vitro activity of extracts and purified compounds on *H. contortus*

Extract	Plant family	Plant species	Plant part	L4 development (IC_{50} in µge/µl) (7 days)
Cn-L	Lauraceae	*Cryptocarya novoguineensis*	Leaves	1.61 ± 0.02
Cn-R	Lauraceae	*Cryptocarya novoguineensis*	Roots	1.55 ± 0.03
Pm-R	Piperaceae	*Piper methysticum*	Roots	0.16 ± 0.06

Potency and cytotoxicity of compounds

Compound	L4 development (IC_{50} in µM) (7 days)	L4 motility (IC_{50} in µM) (72 h)	Toxicity for MCF10A cells (IC_{50} in µM)	Selectivity index (SI)	
				L4 development (7 days)	L4 motility (72 h)
GNT	200–300[a]	6.25–12.5[a]	< 1	< 1	< 1
DHK	207 ± 0.15	No dose-dependent activity	> 300	> 1.50	Not determined
DMY	31.7 ± 0.23	No dose-dependent activity	> 100	> 3.15	Not determined
YGN	23.7 ± 2.05	No dose-dependent activity	45.8 ± 2.74	1.93	Not determined
Control					
MON	0.20 ± 0.01	0.1[a]	26.0 ± 8.06	130	260
MOX	0.08 ± 0.04	0.003 ± 0.01	na	na	na

Not applicable = na.

[a] Estimated from the graphs in Fig. 2.

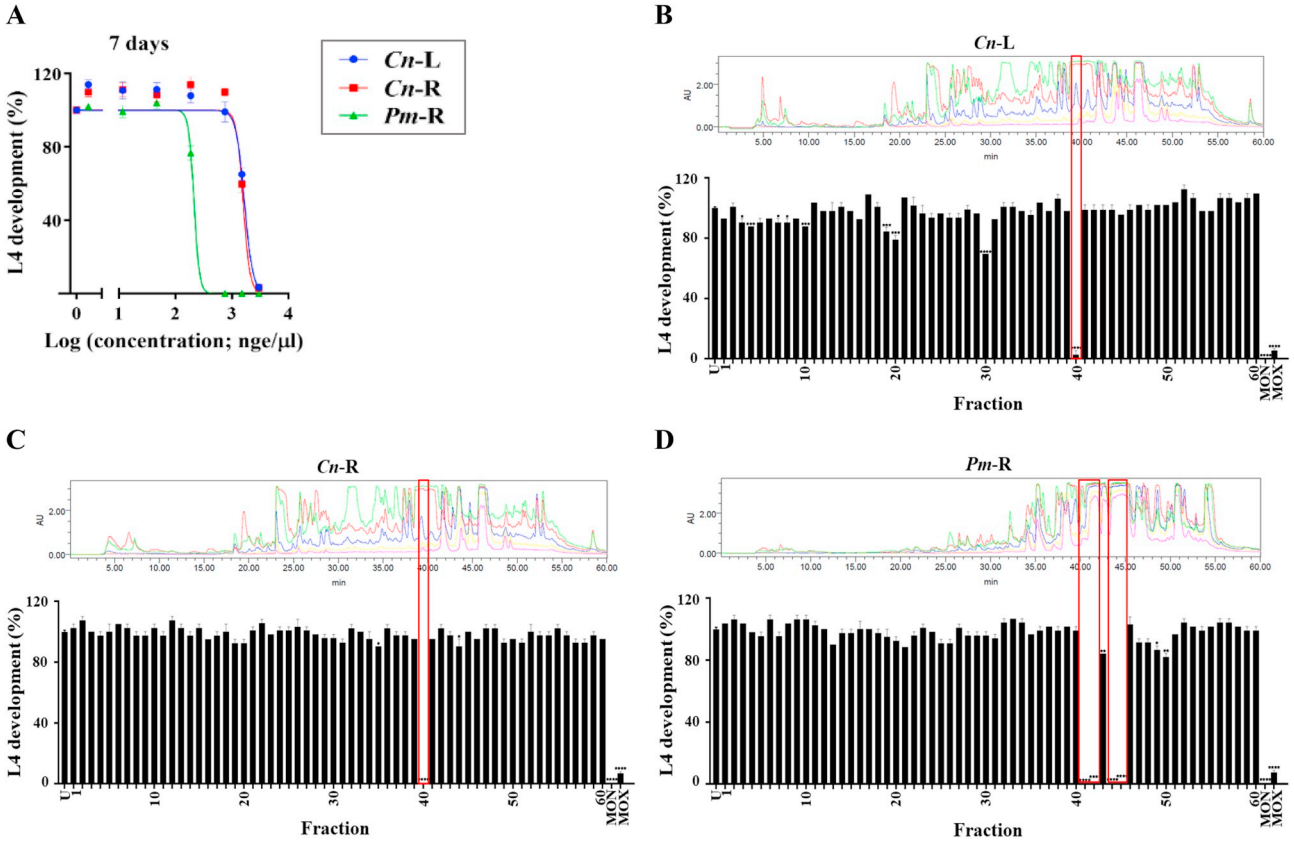

Fig. 1. Effects of extracts and fractions on inhibition of L4 development. Dose-response curves for extracts of leaves (*Cn*-L) and roots (*Cn*-R) from *Cryptocarya novoguineensis* and roots (*Pm*-R) from *Piper methysticum* on L4 development (panel A). Chromatograms and biological activity (L4 development inhibition) of semi-preparative C_{18}-HPLC fractions from three extracts, and of monepantel (MON) and moxidectin (MOX), were compared with untreated (U) controls (i.e. LB* medium +1.25% DMSO). Fraction 40 was the most active in extracts *Cn*-L and *Cn*-R (panels B and C), and four fractions, 41–42 and 44–45 had the highest biological activity in extract, *Pm*-R (panel D). Fraction/s with the highest biological activity are indicated by red rectangles in panels B–D. Asterisks indicate significantly different L4 development compared with the untreated control (****$P < 0.0001$, ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

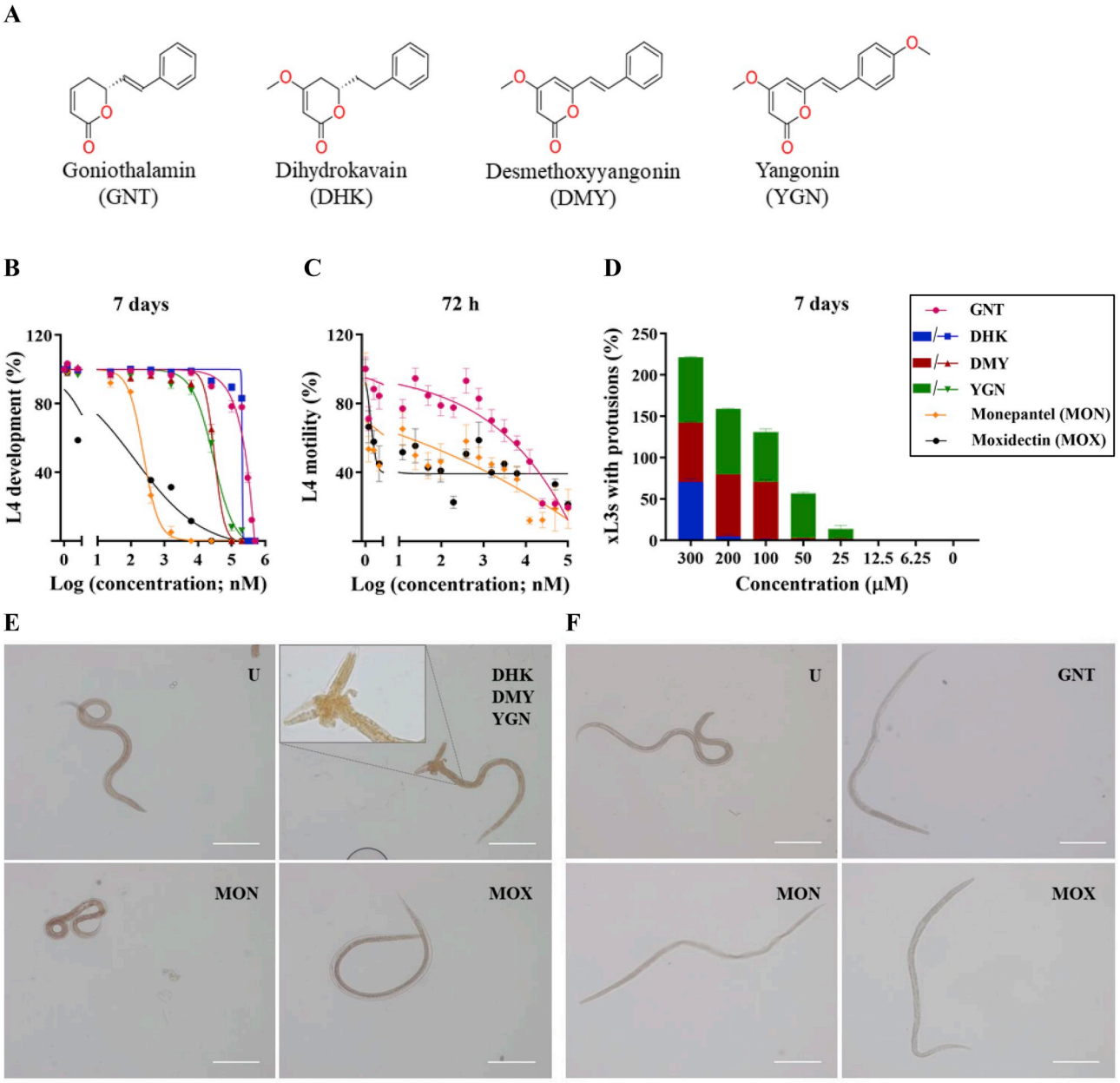

Fig. 2. Chemical structures and in vitro activity of purified compounds on larval stages of *Haemonchus contortus*. Chemical structures of four α-pyrones purified from two plant species, goniothalamin (GNT) from *Cryptocarya novoguineensis*, and dihydrokavain (DHK), desmethoxyyangonin (DMY) and yangonin (YGN) from *Piper methysticum* (panel A). Dose-response curves for purified α-pyrones on L4 development (panel B), and for GNT on L4 motility at 72 h (panel C). The percentages of xL3s with an evisceration (Evi) phenotype after incubation with each of three kavalactones (i.e. DHK, DMY and YGN) at different concentrations (0–300 μM) for 7 days (panel D). Representative light microscopy images of xL3s treated with compounds, DHK, DMY and YGN showing the Evi phenotype of xL3s compared with unaffected larvae in the untreated (U) control, and 'coiled', cuticular-damaged xL3s treated with the control compounds monepantel (MON) and moxidectin (MOX) (panel E). The 'straight', relatively thinner and cuticular-damaged L4s treated with GNT, MON or MOX controls by comparison with relatively thicker, undamaged L4s in the untreated (U) control (panel F); white scale bar: 100 μm; 20× magnification. The sub-image (panel E) showing the protrusion at the anterior part of the xL3 at 100× magnification.

(Fig. 2). Inspection of the dose-response curve of this compound indicated that the IC_{50} was 6.25–12.50 μM at 72 h (Fig. 2; Table 1). The other compounds, DHK, DMY, YGN (from *P. methysticum*), did not inhibit L4 motility, even at the highest tested concentration of 500 μM.

3.4. Evisceration (Evi) phenotype observed in xL3s treated with compounds from P. methysticum

A lethal morphological phenotype was identified by light microscopy (100× magnification) in xL3s treated with individual compounds DHK, DMY and YGN purified from *P. methysticum* (Fig. 2), but not with GNT, even at 500 μM, the concentration at which the compound inhibited L4 development by 100%. This phenotype has been described previously as an initial anterior protrusion followed by evisceration (Evi) (Jiao et al., 2018). For both DMY and YGN, the lowest concentration to induce the Evi phenotype was 12.5 μM (at 7 days), while it was 50 μM for compound DHK (Fig. 2). For all three kavalactones, the potency at eliciting the Evi phenotype related to the potency to inhibit L4 development. For instance, the highest percentages of Evi xL3s were observed at concentrations that inhibited ≥80% of L4 development

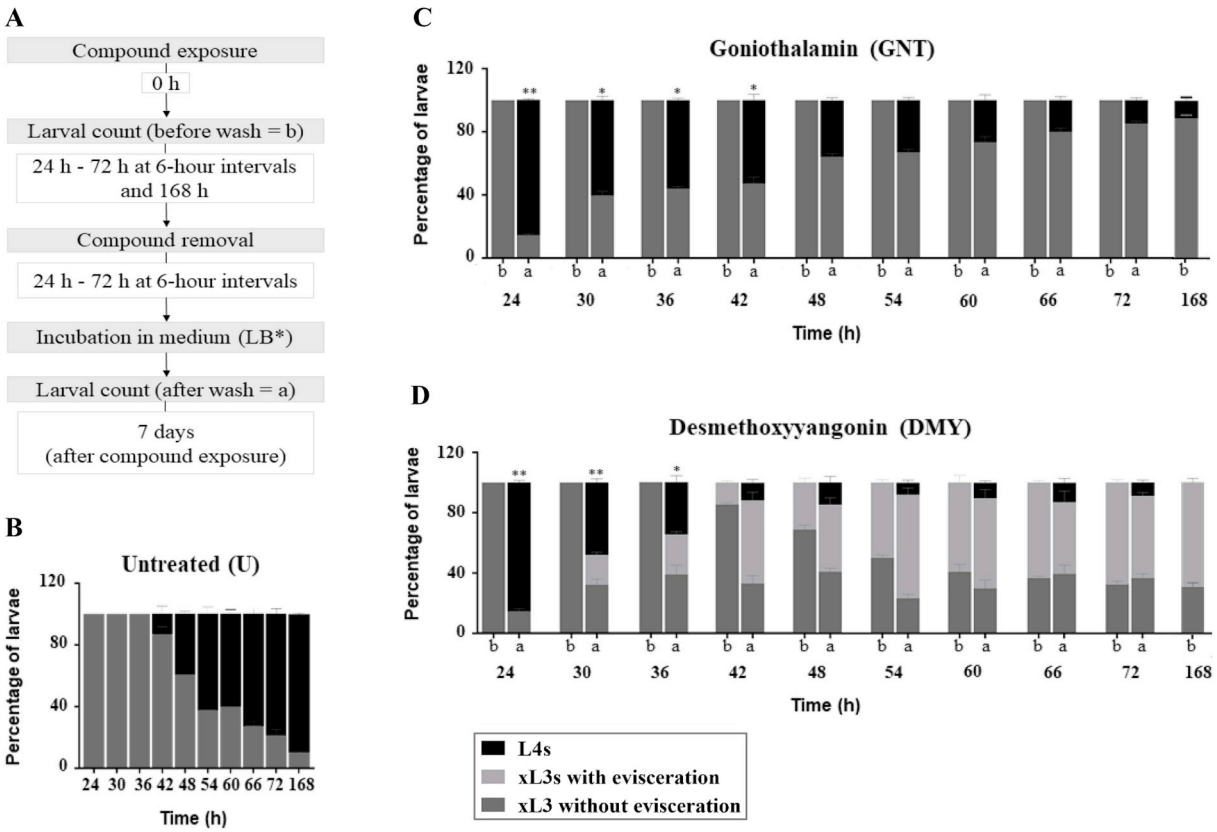

Fig. 3. The irreversibility of compound action. Experimental design (panel A). Bar graph showing the percentages of xL3s and L4s at different time points during the 7-day incubation of untreated (U) larvae (panel B). Bar graphs showing the percentages of xL3s with and without an evisceration (Evi) phenotype, and L4s at different time points of incubation with the compound (before wash = b), and the larval percentages at 7 days after removal of the compound at different time points (after wash = a) for goniothalamin (GNT) (panel C) and desmethoxyyangonin (DMY) (panel D). Compounds, GNT and DMY were tested at concentrations of 400 μM and 100 μM, respectively. Asterisks indicate significantly different L4 development compared with the continually-exposed (i.e. unwashed) control (168 h, b) (**$P < 0.01$,*$P < 0.05$).

(300 μM for DHK, 100 μM for DMY and 50 μM for YGN; see Fig. 2). In addition, a time-course experiment showed that 30 h of exposure to DMY was sufficient to induce the Evi phenotype (scored at 7 days), even though the first Evi larvae were not observed until 42 h (see Subsection 3.5; Fig. 3).

3.5. Irreversible effects of the compounds on larvae

The irreversibility of the effects of compounds GNT and DMY on xL3s was evaluated. The latest recorded time points to allow significant ($P < 0.05$) larval recovery (i.e. L4 development) following the removal of GNT and DMY compared with continually-exposed, matched controls were 42 h and 36 h, respectively (Fig. 3). Therefore, the shortest recorded times for GNT and DMY to exert irreversible effects similar to those of continually-exposed controls were 48 h and 42 h, respectively (Fig. 3).

3.6. Cytotoxicity and selectivity of compounds

All four α-pyrones (GNT, DHK, DMY and YGN) were individually tested for their toxicity on MCF10A cells. The three kavalactones (DHK, DMY and YGN) had relatively low toxicities (IC_{50} values of > 300 μM, > 100 μM and 45.8 ± 2.74 μM, respectively) compared with those of GNT (IC_{50} < 1 μM) and the controls, doxorubicin (0.002 ± 0.00 μM) and monepantel (26.0 ± 8.06 μM) (Table 1). While GNT was not selective at inhibiting either development or motility of the larval stages of *H. contortus* tested, the SIs for three kavalactones for the inhibition of L4 development ranged from 1.5 to 3.2 (Table 1).

4. Discussion

In vitro-screening of all 7500 plant extracts from the NatureBank library identified three extracts from the Papua New Guinean plants *Cryptocarya novoguineensis* and *Piper methysticum* with significant anthelmintic activity against *H. contortus*. The bioassay-guided selection of anthelmintic fractions from these extracts and subsequent purification/analysis thereof identified four compounds with an α-pyrone scaffold, namely GNT (goniothalamin) from *C. novoguineensis* as well as DHK (dihydrokavain), DMY (desmethoxyyangonin) and YGN (yangonin) from *P. methysticum*.

Both *C. novoguineensis* and *P. methysticum* are reported to have numerous medicinal applications. For example, *C. novoguineensis* extracts have been used in South-East Asia to treat fever and diarrhoea (Kostermans, 1989; Allen et al., 2009), and GNT, the bioactive compound purified in this study, is known for its anti-cancer activity induced via apoptosis, genotoxicity and/or antiproliferative effects (Chien and Pihie, 2003; Chan et al., 2010; Al-Qubaisi et al., 2011; Souza et al., 2012; Semprebon et al., 2014). In addition, GNT has been reported to possess anti-bacterial (Mosaddik and Haque, 2003), antifungal (Mosaddik and Haque, 2003), anti-trypanosome (de Fatima et al., 2006a) and anti-malaria properties (Mohd Ridzuan et al., 2006) and, interestingly, anthelmintic activity against the root-knot nematode *Meloidogyne* (Campos et al., 2016). Similarly, *P. methysticum* (kava plant) has been used in the Western Pacific Islands, for thousands of years, to prepare a “social” beverage with psychotropic (anxiolytic, sedative or hypnotic) effects (Singh, 1992). These characteristic effects facilitated the entry of kavalactone products into the commercial market; kavalactones can be found as ingredients of “over-the-counter”

products in some countries including Australia (Sarris et al., 2011), although they are banned in some countries due to their reported, but rare hepatotoxicity (Teschke et al., 2003, 2008). Apart from their anxiolytic properties, kavalactones have been reported to also exert antiviral (Li et al., 2017) and anti-cancer (Li et al., 2012) effects. Interestingly, the present study is the first report of activity of GNT and kavalactones against a parasitic nematode of animals.

The pronounced inhibitory effects of unfractionated extracts on the motility of *H. contortus* xL3s, which were not observed for individual fractions or compounds, might be associated with synergistic effects among compounds present at relatively high concentrations in the extracts. However, the inhibition of L4 development was distinctive and consistent during the fractionation process, and was thus used to guide the selection of anthelmintic fractions and compounds. Here, we showed that all three kavalactones induced the Evi phenotype in treated xL3s and, thus, eliminated further development, but none of the three inhibited L4 motility. The absence or the non-essentiality of the target (present in xL3s) for the survival of developing L4s, or differences in pharmacological properties (e.g., permeability or metabolism) of the compounds between the two developmental stages of *H. contortus* might explain the lack of activity of kavalactones on the L4 stage. Despite the low potency of GNT at inhibiting L4 development, it was the only compound that suppressed L4 motility at a relatively high potency. This differential activity of GNT might be associated with distinct levels of target expression, with variable pharmacokinetic properties of the compound between the two larval stages and/or with an efficient drug-uptake by the L4 stage due to its well-developed mouth and pharynx. As GNT affected L4 motility but did not induce the Evi phenotype in xL3s, it seems reasonable to assume that this compound binds to, or modulates, one or more targets that is/are distinct from that/those of kavalactones. However, given the structural similarity of these compounds, we suggest that the presence of the methoxy group of the pyrone ring of kavalactones might be a determinant for the induction Evi phenotype in xL3s, while its absence allowed GNT to affect L4 motility.

As there was a marked reduction in L4 development (7 days) after removal of GNT at 48 h and of a representative kavalactone (DMY) at 42 h, we propose that these compounds either irreversibly bind to their respective targets or cause irreparable damage to larvae. However, this hypothesis needs to be tested via future molecular and/or biochemical investigations. In the current study, GNT was shown to be substantially more toxic in vitro to MCF10A cells than kavalactones. This finding contrasts the results of a previous study (Punganuru et al., 2018) showing that GNT was not toxic to the same cell-line using a resazurin reduction assay, which assessed metabolic activity rather than cell proliferation (as measured in the present study). Notably, the methoxy substituent on the phenyl ring is the only structural difference between the two equipotent, closely related analogues, DMY and YGN, which exerted different levels of toxicity to MCF10A cells. Thus, the substitution at this position should be considered in future medicinal chemistry optimisation of this scaffold.

The molecular targets of both GNT and kavalactones in *H. contortus* are presently unknown. However, hypotheses regarding targets have been formulated for these compounds in mammals. For instance, GNT is predicted to act on targets including the proteases cascade (caspases 3 and 9), cytochrome *c*, protein kinase A and the mitochondrial permeability transition pore complex (de Fatima et al., 2006b), whereas the targets proposed for kavalactones include GABA (aminobutyric acid) and benzodiazepine receptors, voltage-gated ion channels, arachidonate cascade and monoamine oxidase (Ligresti et al., 2012). These proposals encourage future investigations to identify the targets of these α-pyrones and their modes of action in *H. contortus* and other nematodes.

In conclusion, the bioassay-guided fractionation of the extracts of two plant species, *C. novoguineensis* and *P. methysticum*, and further purification yielded four known α-pyrones with anthelmintic activity against *H. contortus*. Given the ability of kavalactones to induce a lethal phenotype in xL3s and the relatively potent activity of GNT against the L4 stage, these compounds are considered as valid chemical starting points for the development of novel anthelmintics. Their simple drug-like structures should facilitate chemical development and their evaluation as potential drug leads, reinvigorating work on natural products as sources of anti-parasitic agents.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijpddr.2018.12.006.

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