Eryngial (*trans*-2-dodecenal), a bioactive compound from *Eryngium foetidum*: its identification, chemical isolation, characterization and comparison with ivermectin *in vitro*

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SUMMARY

Methanol-water (4:1, v/v) crude extracts (50 mg mL^{-1}) of 25 Jamaican medicinal plants were screened *in vitro* for anthelmintic activity using infective third-stage larvae of *Strongyloides stercoralis*. The most effective extract was further chemically scrutinized to isolate and identify the source of the bioactivity, and the efficacy of this compound was compared with ivermectin. Eosin exclusion (0·1 mg mL^{-1}) served as the indicator of mortality in all bioassays. A crude extract of *Eryngium foetidum* (Apiaceae) was significantly (Probit Analysis, $P<0·05$) more potent than the other plant extracts, taking 18·9 h to kill 50% (LT_{50}) of the larvae. Further, the petrol extract of *E. foetidum* was significantly more effective (Probit Analysis, $P<0·05$) at killing the larvae (LT_{50}, 4·7 h) than either its methanol–water or dichloromethane extract. The latter two effected less than 1% larval mortality after 120 h. With bioassay-driven column chromatography of the petrol extract, *trans*-2-dodecenal (eryngial) was identified and chemically isolated as the main anthelmintic compound in *E. foetidum*. There was a significant difference between the 24 h LD_{50} values (mM) of *trans*-2-dodecenal (0·461) and ivermectin (2·251) but there was none between the 48 h LD_{50} values (mM): *trans*-2-dodecenal (0·411) and ivermectin (0·499) *in vitro*.

Key words: Medicinal plants, *trans*-2-dodecenal, ivermectin, bioassay, *Strongyloides stercoralis*.

INTRODUCTION

Strongyloides stercoralis is a common, gastrointestinal parasitic nematode that naturally infects humans, other primates and canines by skin contact (Nolan *et al*. 2011). As many as 200 million people may be infected worldwide (Despommier *et al*. 2005), primarily in tropical and subtropical regions (Garcia and Bruckner, 1988). In the Caribbean, where the infection is endemic (Tikasingh, 1981), with prevalence ranging from 0·1 to 23·6%, strongyloidosis is clinically the most severe nematodiasis. Chronic infections in immunocompetent individuals are typically asymptomatic, and can persist upwards of 30 years as a result of autoinfection (Gill and Bell, 1979; Grove, 1980). However, hyperinfections can occur and are linked to poor nutrition and immunosuppression caused by illness and/or corticosteroid treatment. The resulting overwhelming, or disseminated, strongyloidosis is often fatal (Cruz *et al*. 1966; Grove 1980; Powell *et al*. 1980; Ingra-Siegnam *et al*. 1981; Davidson *et al*. 1984; Genta, 1989; Mansfield and Schad, 1992).

With transmission of *S. stercoralis* enhanced by poor sanitary conditions in the surroundings of infected individuals and its ability to persist in the soil under warm, moist conditions (Grove, 1980), an effective control strategy against the disease has been met with challenges of diagnosis (Siddiqui and Berk, 2001) and treatment (Terry, 1987). Commercial drugs developed for treating strongyloidosis exhibit unreliable effectiveness, and have other associated problems. For example, of the benzimidazoles, thiabendazole [2-(4′-thiazoyl)-benzimidazole] (Mintezol; Merck, Sharp & Dohme) was the drug of choice for treating chronic and severe strongyloidosis in the Caribbean (Grove, 1982*b*), but poor efficacy, toxicity, unpleasant side effects, and high costs have deterred its use (Bezjak, 1968; Gustaffson *et al*. 1987; Terry, 1987). Despite some success in-treating strongyloidosis, albendazole [5-(propylthio)-2-carbomethoxyamino-benzimidazole] (Zentel; Smith, Kline Beecham) is associated with teratogenicity and embryotoxicity in experimental animals (Gustafsson *et al*. 1987). Thus, its use has been discouraged, especially in women during pregnancy and lactation. Mebendazole [methyl 5-benzoyl-2-benzimidazole carbamate] (Vermox; Janssen) was reported to be inactive against the infective larvae in immunosuppressed dogs, and has little effect on the parasite in muscle tissue of mice (Grove, 1982*a*). Use of cambendazole ([5-isopropoxycarbonylamino-2-(4-thiazyl) benzimidazole] (Merck, Sharp & Dohme) has been banned as a result of its linkage to several

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fatal idiosyncratic reactions in cattle (Hogg, 1978; Main and Vass, 1980).

Alternatively, ivermectin [22,23-dihydroavermectin B_1] (Merck, Sharp & Dohme), a broad-spectrum, commercial drug (Fisher, 1985; Grove, 1989) that is a synthetic, lipophilic, macrocyclic lactone (Gustafsson *et al.* 1987; Campbell, 1989), bears promise as an efficacious treatment against strongyloidosis (Naquira *et al.* 1989; Marti *et al.* 1996). Cure rates of 82–88% have been reported (Naquira *et al.* 1989; Adenusi *et al.* 2003). However, more work is needed to further confirm its efficacy (Adenusi *et al.* 2003), and there are reports of emerging drug resistance against ivermectin by parasitic nematodes (Echevarria and Trindade, 1989; Jackson and Coop, 2000; Currie *et al.* 2004), including *S. stercoralis* (Gill *et al.* 1991; Shikiya *et al.* 1992). Additionally, in Jamaica where the majority of infected individuals are of poor socio-economic status, the suggested single-dose regimen for ivermectin therapy (200 μg kg^{-1} body weight) at an average cost of US$32.00, is often unaffordable.

This paucity of effective drug therapy against strongyloidosis highlights the imperative for new sources of treatment that are efficacious and affordable.

Since time immemorial, medicinal plants have been used almost entirely by large percentages of human populations in the Caribbean, Africa, Asia and other tropical regions in the form of concoctions and decoctions for treating a myriad of ailments including intestinal worm infections (Ayensu, 1981; Dagar and Dagar, 1991; Naranjo *et al.* 1995). Although there is abundant information on folklore usage of plants across the world, more effort is needed to scientifically validate these practices and folk-claims. Since Sloane's account (Sloane, 1725) about 60 publications have appeared on the topic of folklore medicine in the Caribbean with only a few dealing with parasitic infections (Asprey and Thornton, 1953; Campbell, 1974; Lal *et al.* 1976; Goulart *et al.* 1977; Ayensu, 1981; Robinson *et al.* 1990). Thus, in the search for a new, safe and effective treatment against strongyloidosis, Jamaican medicinal plants were selected based on their folklore usage against intestinal worm infections. Some were also selected on the basis of insecticidal activity, because similarities exist in neuromuscular transmission between nematodes and insects (Leake and Walker, 1980), while others were selected based on known chemotaxonomic properties of allied species (Trease and Evans, 1934; Williams, 1992; Albert and Struwe, 1997).

MATERIALS AND METHODS

Test organism

Infection of *S. stercoralis*, procured from a 57-year-old female patient in Jamaica, was established in two parasite-free mongrel dogs of the same litter by cutaneous inoculation of each in the mid-dorsal neck region with 1 mL Locke's nematode saline (LNS) containing 3000 infective larvae (L3i) (Genta, 1989). First-stage rhabditiform larvae (L1) were first detected at 53 and 70 days post-larval inoculation in the dogs. Infections were maintained by administering prednisolone (Predol retention enema, Glaxo Australia Pty. Ltd., Boronia, Vic.) daily at 0·5 mg kg^{-1} *per os* dog (Dawkins and Grove, 1982; Schad *et al.* 1984), under professional veterinary supervision. The dogs were fed and watered *ad libitum*. L3i 5 days old were harvested from charcoal coprocultures in LNS and used as the test organisms in all bioassays. They were gravimetrically quantified using a counter and an Alphaphot 2 compound light microscope (Nikon) so that 20 μL sub-samples contained about 50 L3i.

Assessment of larval mortality by eosin and determination of optimal concentration

The dye-exclusion technique (Halton and Arme, 1971; Robinson and Arme, 1985) was used to indicate larval mortality in all bioassays. Heat-killed (test) as well as live larvae (control) were exposed to eosin (0·1, 0·3 and 1 mg mL^{-1}) in LNS. Counts of stained and non-stained larvae in 20 μL suspensions were conducted at 10 min intervals for 1 h. Optimal eosin concentration as an indicator of larval mortality was determined by exposing live specimens to eosin (0·1, 0·3 and 1 mg mL^{-1}) and counting the number of mobile, immobile (not stained) and dead (stained) larvae at 0, 1, 2, 3 and 6 h post incubation, and subsequently at 24 h intervals for 96 h.

Plant selection

Twenty-five plant species were selected for crude extract preparation using either whole plants or specific plant tissues such as leaves, roots, stem and/or flowers. *Andrographis paniculata, Centrostachys indica, Allium sativum, Eryngium foetidum, Tamarindus indica, Carica papaya, Chenopodium ambrisoides, Ambrosia hispida, Bidens pilosa, Cucurbita pepo, Ricinus communis, Rytidophyllum tomentosum, Aloe vulgaris, Cecropia peltata, Ravina humilis, Portulaca oleracea, Punica granatum, Picrasma excelsa* and *Stachytarpheta jamaicensis* were selected on the basis of their usage in Caribbean folklore against intestinal worms (Asprey and Thornton, 1953; Adams, 1972; Campbell, 1974; Honychurch, 1980; Ayensu, 1981). *Annona muricata* and *Annona squamosa*, which are also used in Caribbean folklore, were chosen based on demonstrated chemotaxonomic properties of allied species (Waterman, 1986). Finally, *Cuscuta americana, Azadirachta indica, Mimosa pudica* and *Artocarpus altilis*, besides their folklore medicinal uses, also have known insecticidal

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properties (Zanno *et al*. 1975; Jacobson, 1981, 1986; Williams, 1992; Wilson, 1993).

Preparation of crude extracts

Each green plant (20 g) was chopped and extracted with 0·1 L methanol–water (4:1, v/v) for 5 days at room temperature. Crude extracts were washed with methanol–water (5 mL) twice and filtered. The filtrates were pooled, concentrated *in vacuo* and freeze-dried (3 × 24 h) to remove water and methanol residue (see determination of methanol content). Freeze-dried crude extracts were weighed and their yields (g) determined. All extracts were stored at −4 °C before bioassay.

Determination of methanol content in crude extracts

Carbon-13 (^{13}C) Nuclear Magnetic Resonance (NMR) spectral analysis was used to detect methanol residue in randomly selected *R. communis, A. cepa* and *S. jamaicensis* crude extracts (100 mg) before and after freeze-drying. These were dissolved in deuterium oxide (3 mL) and filtered. ^{13}C NMR spectral analyses of the extracts were determined on a Bruker AC 200 instrument. Sodium trimethylsilylpropanoate (TSP) was used as the internal standard. Methanol concentrations [μg CH_3OH/100 mg extract] were approximated by comparing relative differences of NMR signals (^{13}C peak heights) with that of the TSP (internal) standard.

Assessment of larval tolerance to methanol

Due to the toxicity of methanol (Cornish, 1975), and its detection in freeze-dried samples, tolerance of infective larvae to the solvent was investigated in triplicate at 0 (control), 1·0, 5·0 and 10·0 μg CH_3OH/100 mg in LNS in the presence of eosin (0·1 mg mL^{-1}). The bioassay was conducted as indicated below (bioassay of crude plant extracts).

Bioassay of crude plant extracts

A stock suspension (100 mg mL^{-1}) of each crude plant extract that contained eosin (0·2 mg mL^{-1}) was prepared in LNS. An equal volume of LNS with L3i was added to a known volume of the stock, thereby halving its concentration along with eosin, and adjusting the number of L3i in sub-samples (20 μL) to about 50 L3i. Anthelmintic potential of each plant extract (50 mg mL^{-1}) was investigated *in vitro* by counting the number of L3i that were mobile, immobile (not stained) and dead (stained with eosin) at 0, 1, 2, 3 and 6 h post-larval incubation, and at subsequent 24 h intervals for 5 days in tests and controls (triplicate). The larvae were kept in a light-free environment between counts to preserve their viability. Relative activity (RA) was calculated by dividing LT_{50} of the most effective extract (shortest duration to kill 50% of the L3i) by the LT_{50} of each extract. With *E. foetidum* crude extract being the most potent, a specimen of the plant with the assigned accession number 33 700 was deposited in Botany Herbarium of The University of the West Indies, Mona Campus, Jamaica, West Indies.

Preparation of E. foetidum *crude extracts*

Whole, green plants of *E. foetidum* (2·46 kg) were chopped and extracted with petrol (6 L) for approximately 36 h to yield an insoluble, oily extract (2·624 g) with a characteristic pungent odour. Petrol refers to the petroleum fraction with boiling point 60–80 °C. Plant extraction was continued with dichloromethane (5·5 L) for 48 h to yield a greenish-brown insoluble residue (5·43 g). Finally, the plant material was extracted with methanol–water (4:1), which yielded a dark brown extract (5·764 g) after freeze-drying (3 × 24 h).

Bioassay of emulsifier treatments

With the petrol and dichloromethane crude extracts of *E. foetidum* being water insoluble, an effective emulsifier to homogenize the extracts was identified accordingly. Triton X-100 and 'Tween 20' (1, 2 and 4 mg mL^{-1}) in LNS with eosin (0·1 mg mL^{-1}) were bioassayed in triplicate, respectively to assess for any larval inhibitory effect by the emulsifiers. Counts of larvae in 20 μL sub-samples were done at the same time intervals stated above. Controls had no emulsifier. With the inability of Triton X-100 (1, 2 and 4 mg mL^{-1}) to adequately dissolve *E. foetidum* dichloromethane and petrol crude extracts, higher concentrations (10, 20 and 40 mg mL^{-1}) were similarly bioassayed.

Bioassay of E. foetidum *crude extracts*

Stock suspensions of *E. foetidum* petrol, dichloromethane and methanol:water crude extracts (100 mg mL^{-1}) in LNS with Triton-X 100 (20 mg mL^{-1}) and eosin (0·2 mg mL^{-1}) were diluted to 50 mg mL^{-1} by adding equal volumes of LNS that contained L3i and then were bioassayed. Larval mortality was assessed by counting the L3i in homogeneous sub-samples (20 μL) at similar time intervals that were used for the 25 plant crude extract bioassays.

Test for the presence of triolein and stigmasterol in E. foetidum *petrol crude extract, and separation and demarcation of its compounds by column and thin layer chromatography*

The petrol extract of *E. foetidum* was examined for the presence of triolein and stigmasterol using thin

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layer chromatography (TLC). The extract (1·005 g) was also subjected to TLC and gravity column chromatography, which involved the use of silica gel (230–400 mesh, 40–63 μm in diameter, Sigma) as the stationary phase and increasing concentrations of ethyl acetate in petrol as the mobile phase. Polyester backed pre-coated silica gel plates $HF_{254+366}$ (0·25 mm thickness, 20 × 20 cm, Sigma Aldrich) with fluorescent indicator were used for TLC work. 10 mL aliquots were collected under gravity and the eluents were subjected to TLC and UV analyses to assess the separated compounds. Chromatograms were compared with the petrol crude extract and aliquots containing compounds having similar R_f values were pooled and visualized by spraying with dodecamolybdophosphoric acid/ceric sulphate spray reagent and viewing under a UV lamp for active compounds. Pooled aliquots were concentrated *in vacuo* and yields (g) of the compounds were determined.

Bioassay to further identify locality of active compound(s) in the petrol crude extract

Triplicate 20 mg mL^{-1} suspensions of eight pooled 10 mL column chromatography aliquots that contained 16 compounds in the *E. foetidum* petrol crude extract were prepared in LNS. These also contained 20 mg mL^{-1} Triton X-100 and eosin (0·2 mg mL^{-1}). Equal volumes of LNS with L3i were added, thereby halving concentrations of the compounds, emulsifier and eosin. Counts of larvae in 20 μL sub-samples were carried out at the same time intervals used for crude plant extracts. The bioassays allowed for further identification of the locality of the active compound(s), based on the aliquots that inactivated the larvae.

Purification of the anthelmintic compound, trans*-2-dodecenal*

Having identified the pooled aliquots of the *E. foetidum* petrol crude extract with greatest anthelmintic activity, purification was effected by chromatography of the extract (434 mg) using four 20 × 20 cm preparative thin layer chromatographic plates (0·5 mm thickness). The solvent system used consisted of ethyl acetate: petrol: acetic acid in a 4:96:1 ratio. The plates were run once in the solvent system to give two main bands with R_f values of 0·12 and 0·76. The compounds were visualized by spraying with a 2,7-dichlorofluorescein solution followed by observation under a UV lamp. The high R_f band contained the compound of interest. Therefore, a second set of two plates utilizing 2% ethyl acetate in petrol as the eluent was run for further purification. This purification attempt yielded 77·0 mg of *trans*-2-dodecenal (eryngial). This was analysed by infrared, NMR and mass spectroscopy.

Bioassay of the anthelmintic compound, trans-2-dodecenal, and its comparison with ivermectin

The 24 and 48 h 50% lethal doses (LD_{50}) of eryngial and ivermectin (mM) were investigated *in vitro* using the L3i. Ivermectin was obtained from Sigma Aldrich, St. Louis, MO. The larvae were exposed to a range of dilutions of the compounds by adding known volumes of LNS with L3i to known volumes of stock suspensions of the anthelmintic compounds (10 mg mL^{-1} in LNS) which contained final concentrations of Triton X-100 (2 mg mL^{-1}) and eosin (0·1 mg mL^{-1}). Each dilution, including the control (0 mg mL^{-1}), was prepared in triplicate. 20 μL sub-samples contained about 50 L3i. Larval mortality was assessed in a similar way to the plant crude extract bioassays.

Treatment of data

All data were corrected for control mortality using Abbot's formula (Busvine, 1972):

Abbot's formula:

$$P = \frac{P_1 - C}{100 - C} \times 100$$

where, P = corrected percentage inactivation; P_1 = observed percentage inactivation; C = control percentage inactivation.

The time (h) taken by each crude extract to kill 50% of the infective larvae (LT_{50}) was determined using Probit Analysis (Busvine, 1972). Fiducial limits could not be calculated from the data whenever the heterogeneity factor (g) exceeded 0·5 (Finney, 1975). In such instances, Analysis of Covariance was used to test if significant differences existed between slopes of the control and test solutions. Probit analysis was also used to determine the 24 h LD_{50} values of *trans*-2-dodecenal and ivermectin *in vitro*.

RESULTS

Indicator of larval mortality and tolerance to eosin

Eosin (0·1, 0·3 and 1 mg mL^{-1}) in LNS indicated staining (mortality) of heat killed L3i after 10 min of exposure, starting at the oesophagus and proctodeum. Staining was not observed in the live control larvae. The eosin concentrations also resulted in about 2% larval mortality (cumulative corrected percentage) after 96 h. Eosin (0·1 mg mL^{-1}) was hence used as the indicator of larval mortality in all *in vitro* bioassays because it was the lowest effective concentration.

Methanol content in crude extracts

Methanol concentrations (μg/100 mg) after freeze-drying (24 h) extracts of *R. communis*, *S. jamaicensis* and *A. cepo* were 0·1, 0·025 and 0·1, respectively.

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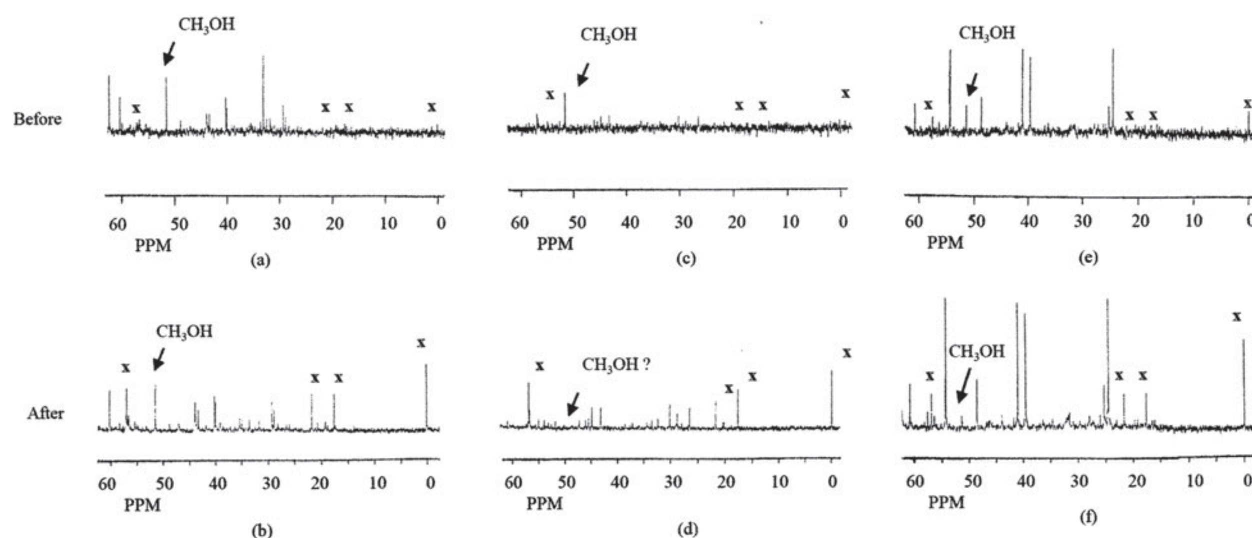

Fig. 1. Partial ^{13}C NMR spectrum of methanol–water (4:1) crude extracts of *R. communis, A.cepa* and *S. jamaicensis* (solvent, D_2O) before refreeze-drying (a, c and e), and after refreeze-drying (b, d and f), respectively. X denotes signals from internal standard (TSP). NB: unmarked peaks represent other compounds in the plant extract.

Table 1. Concentration of methanol in crude extracts detected by nuclear magnetic resonance (NMR) analyses before and after additional 48 h freeze-drying (FD)

Plant	(μg CH_3OH/100 mg crude extract) before 48 h FD	(μg CH_3OH/100 mg crude extract) after 48 h FD
R. communis	0·1	0·072
S. jamaicensis	0·025	0·009
A. pepo	0·1	0·01

Methanol concentration [μg CH_3OH/100 mg crude extract] determined by comparison of differences of NMR signals with the standard, sodium trimethylsilylpropanoate (TSP).

With additional freeze-drying (2 × 24 h), methanol concentration in the extracts was reduced by 1·4, 2·8 and 10·0 fold, respectively (Table 1). Nuclear magnetic resonance spectra indicated reduced levels of methanol in the crude extract samples before and after refreeze-drying (Fig. 1). Methanol concentrations were calculated based on signal intensities in the spectra. The other crude extracts were similarly refreeze-dried and for the same duration (2 × 24 h).

Larval tolerance to methanol

Analysis of Covariance revealed no significant difference between slopes of the control and test solutions of methanol for larval mortality, except that the slope of the 10 μg CH_3OH/100 mg solution was significantly different from those of the control and 1 μg CH_3OH/100 mg solutions ($F = 5{\cdot}868$, $F_{0{\cdot}05\,(3)\,3,\,124} = 2{\cdot}67$, $P < 0{\cdot}05$). Methanol concentrations that were 10, 50 and 100 times greater than the highest concentration detected by NMR analyses (Table 1) had a mortality effect of approximately 10% on the L3i after 120 h. Refreeze-drying further reduced concentrations of methanol in the extracts to less than 0·07 μg CH_3OH/100 mg solution (Table 1).

Larval behavioural response to crude extracts

Strongyloides stercoralis L3i were typically active in control solutions, swimming vigorously in a wiggling motion throughout the 5-day duration of each bioassay. Swimming behaviour was, however, altered in plant extracts with anthelmintic activity. Initially, the L3i were generally as active as the controls. However, with time, the darting swimming manoeuvres slowed. Eventually, the larvae stopped swimming and became immobile. Immobility was occasionally punctuated with 'jerky' movements. L3i mortality, which was indicated by eosin staining, was always preceded by permanent immobilization. The rate at which the sequence of events occurred, and the number of larvae that were immobilized only or killed, varied from one crude extract to another.

Anthelmintic activity of crude plant extracts

Durations (h) taken by the crude extracts (50 mg mL^{-1}) of selected plants to kill 50% (LT_{50}) of *S. stercoralis* L3i are shown in Table 2. The extracts' LT_{50} values ranged from 18·9 h (*E. foetidum*) to 99·2 h (*A. paniculata*). LT_{50} values were not determined for crude extract suspensions of *C. papaya, A. muricata, A. altilis* and *P. granatum*, which resulted in less than 50% mortality after 120 h. *Eryngium foetidum* crude extract was significantly more effective at killing the L3i than the others and was therefore subjected to further scrutiny to elucidate the source of anthelmintic activity.

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Table 2. 50% mortality time (LT_{50}, h) of *S. stercoralis* infective larvae by $50\ mg\ mL^{-1}$ crude extracts of selected Jamaican plants. Relative activity of *E. foetidum* crude extract used as reference for other crude extracts

Plant extract ($50\ Mg\ mL^{-1}$)	LT_{50} (h) ± 95% fiducial limits (FL)	Regression equation ($Y = a + bx$)			Relative activity (RA)
		a	*b*	(S.E.)	
E. foetidum	18·9 (12·5–21·3)	−12·89	9·97	(0·52)	1·00
P. oleracea	31·2 (27·8–34·6)	−10·02	6·70	(0·37)	0·606
C. americana	41·8 (33·9–48·9)	−10·37	6·40	(0·22)	0·452
T. indica	43·3 (29·5–58·0)	−5·55	3·51	(0·30)	0·437
B. pilosa	45·7 (35·2–52·6)	−79·66	47·32	(2·97E+06)	0·414
P. excelsa	49·7 (39·9–59·9)	−5·30	3·13	(0·20)	0·380
C. peltata	51·0 (40·9–57·9)	−8·97	5·25	(0·41)	0·371
R. humilis	51·0 (40·9–59·9)	−6·82	3·99	(0·34)	0·371
M. pudica	51·6 (41·4–59·9)	−7·09	4·14	(0·39)	0·366
A. hispida	54·7 (50·5–58·7)	−19·9	11·40	(0·71)	0·346
R. tomentosum	78·2 (62·4–103·5)	−3·30	1·75	(0·13)	0·242
S. jamaicensis	80·4 (79·2–81·8)	−48·84	28·26	(1·81)	0·235
C. pepo	80·8 (72·4–89·3)	−34·43	18·05	(1·15)	0·234
R. communis	84·2 (69·4–94·1)	−19·24	9·99	(0·76)	0·225
A. indica	88·8 (61·2–159·0)	−2·81	1·44	(0·22)	0·213
C. ambrisoides	91·6 (78·9–104·9)	−23·70	12·08	(1·03)	0·206
A. sativum	92·4 (89·5–95·2)	−9·80	7·53	(0·57)	0·205
C. indica	92·4 (66·6–153·5)	−3·71	1·89	(0·84)	0·205
A. squamosa	92·9 (86·3–100·4)	−6·77	3·44	(0·40)	0·203
A. vulgaris	96·3 (80·9–117·4)	−4·80	2·41	(0·52)	0·196
A. paniculata	99·2 (87·3–111·4)	−10·22	5·12	(0·55)	0·191

RA = LT_{50} of the most effective extract divided by LT_{50} of each extract.

Selection of emulsifier

'Tween' 20 (1, 2 and $4\ mg\ mL^{-1}$) in LNS (with eosin, $0·1\ mg\ mL^{-1}$) was toxic to *S. stercoralis* L3i. Cumulative corrected percentage mortality ranged from 8·1 to 40·8% after 96 h. Additionally, 'Tween 20' did not produce homogeneous dispersions of *E. foetidum* dichloromethane and petrol extracts ($100\ mg\ mL^{-1}$) after shaking (12 h). Similar concentrations of Triton X-100, which caused less than 3% cumulative percentage larval mortality after 96 h, also did not produce homogeneous dispersions of the *E. foetidum* extracts. Higher concentrations of Triton X-100 (10, 20 and $40\ mg\ mL^{-1}$) in LNS with eosin (conc. as above) resulted in less than 2% (cumulative corrected percentage) L3i mortality after 96 h and dispersed both crude extracts in the preparation of 10·0% stock suspensions. Hence, Triton X-100 ($10\ mg\ mL^{-1}$) was selected as the lowest effective concentration for homogenizing *E. foetidum* crude extracts.

Anthelmintic activity of E. foetidum *crude extracts*

The LT_{50} value (4·72 h) of *E. foetidum* petrol extract ($50\ mg\ mL^{-1}$) confirmed it to be significantly greater than that of the other extracts. After 6 h, the petrol extract killed 96% of the L3i, while the dichloromethane and methanol:water crude extracts resulted in less than 1% mortality after 120 h.

Absence of triolein and stigmasterol from E. foetidum *petrol crude extract and anthelmintic activity of its compounds*

Thin layer chromatography analysis revealed the absence of triolein and stigmasterol from the *E. foetidum* petrol crude extract. Pooled aliquots 15–35 resulted in 50% larval mortality after 20·8 h and 85·4% mortality after 48 h, while all the other pooled aliquots (1–2, 3–5, 74–83, 107–163) except for aliquots 36–48 and 49–73, killed less than 1% of the L3i after 120 h, respectively. Aliquots 36–48 and 49–73 killed approximately 21 and 12% of the L3i after 120 h, respectively. Pooled aliquots 15–35 was therefore identified as that portion of the eluent that contained the anthelmintic compound(s) in the greatest concentration. Analysis with TCL revealed the presence of the compound(s) of aliquot 15–35 in aliquots 36–48 and 49–73, albeit in smaller quantities.

'Eryngial', the purified anthelmintic compound from E. foetidum

The *E. foetidum* anthelmintic compound was characterized as a pale, yellow oil with a pungent odour; was shown to be a straight chain, unsaturated aldehyde of chemical formula $C_{12}H_{22}O$; and was assigned the name 'eryngial'. The carbon-carbon double bond was found to be in conjugation with the carbonyl

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Table 3. *In vitro* dose-response data for *S. stercoralis* L3i after 24 and 48 h using *trans*-2-dodecenal and ivermectin

Active compound of anthelmintic agents	Dose–response data	
	24 h $LD_{50} \pm 95\%$ fiducial limits (mM)*	48 h $LD_{50} \pm 95\%$ fiducial limits (mM)*
Trans-2-dodecenal	0·461 (0·433–0·483)	0·411 (0·395–0·428)
Ivermectin	2·251 (2·046–2·503)	0·499 (0·337–0·638)

(mM)* – LD_{50} values listed as millimolar concentrations.

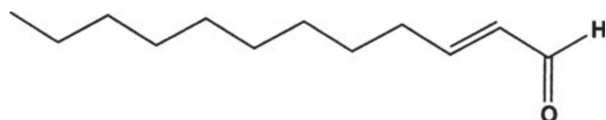

Fig. 2. The chemical structure of *trans*-2-dodecenal ($C_{12}H_{22}O$).

and its configuration was determined to be E. The aldehyde was slowly oxidized to the corresponding carboxylic acid in air. This was confirmed by analysis of the methyl ester of this acid. The preparation of the 2,4-dinitrophenylhydrazone derivative of the aldehyde, as well as Lemieux–von Rudloff degradation to decanoic acid, characterized as its methyl ester, confirmed the structure of eryngial as *trans*-2-dodecenal or *E*-2-dodecenal:

GCMs: m/z (rel. int.) 182·1 (<1) [M^+], 135 (10), 121 (20), 97 (35) [M^+-C_6H_{13}], 83 (76) [M + -C_7H_{15}] (β-cleavage), 70 (98), 55 (80) [C_3H_3O], 43 (70) [C_3H_7], 41 (100), 29 (44) [C_2H_5]; FAB-MS: m/z (rel. int.) 200·5 (61) [M + NH_4^+], 190·4 (14), 81·2 (11), 58·1 (22), 35·1 (100); ^{1}H NMR: δ 0·90 (3H, t, J = 6·80 Hz), 1·31 (12 H, br s), 1·55 (2H, t, J = 7·13 Hz), 2·34 (2H, qd, J = 6·83, 1·44 Hz), 6·13 (1H, ddt, J = 15·83, 7·91, 1·09 Hz), 6·86 (1H, dt, J = 15·83, 6·80 Hz), 9·52 (1H, d, J = 7·91 Hz); ^{13}C NMR: δ 14·0 (CH_3), 22·6 (CH_2), 27·8 (CH_2), 29·16 (CH_2), 29·2 (CH_2), 29·3 (CH_2), 29·4 (CH_2), 31·8 (CH_2), 32·7 (CH_2), 132·9 (CH), 159·0 (CH), 194·1 (CH).

The chemical structure of *trans*-2-dodecenal ($C_{12}H_{22}O$) is shown (Fig. 2).

Bioassay of eryngial and comparison with ivermectin

The order of activity using 24 h LD_{50} (mM) for the anthelmintic compounds was: eryngial, 0·461 > ivermectin, 2·251 (Table 3). The plant compound was significantly more effective at killing the L3i displaying a 4·9-fold increase in activity over ivermectin. There was however, no significant difference between the 48 h LD_{50} (mM) of *trans*-2-dodecenal (0·411) and ivermectin (0·499) (Table 3).

DISCUSSION

This study confirms the role of traditional medicine in the search for bioactive compounds of natural product origin. Folklore-claims, that *E. foetidum* and other selected medicinal plants possess anthelmintic activity, were confirmed by rigorous scientific scrutiny of the extracts using *S. stercoralis* L3i.

For the first time, based on 50 mg mL^{-1} T_{50} values (Table 2), it was shown that over 19 species of Jamaican plants belonging to different families exhibited a spectrum of activity, ranging from those that were strongly anthelmintic to those with little or no inactivation potential. For example, *E. foetidum* crude extract (50 mg mL^{-1}) killed 50% of the L3i after 18·9 h, while *P. granatum* was ineffective at killing the larvae even after 120 h.

Eryngium foetidum crude extract was the most effective at killing the L3i based on the LT_{50} values and was therefore subjected to further scrutiny. For the first time, eryngial was identified and isolated as the main source of anthelmintic activity, and demonstrated acute toxicity against *S. stercoralis* L3i *in vitro*. Localization of eryngial in the plant's petrol crude extract (R_f value = 0·40 in 10% EtOAc/petrol) and its chemical isolation were made possible by systematic bioassay guided fractionation and chromatography techniques.

Standardization of the methodology ensured reliable assessment of the plant extracts' anthelmintic potential. For example, staining of dead *S. stercoralis* L3i, via the eosin exclusion technique (Halton and Arme, 1971; Robinson and Arme, 1985), successfully indicated larval mortality throughout all bioassay work. This was based on the principle that selective cuticular semipermeability to solutes is compromised in dead organisms. The eosin exclusion technique was therefore used to facilitate differentiation of dead larvae from those that were either mobile or immobile but not dead. Hence, LT_{50} values of the crude extracts using *S. stercoralis* L3i larvae could be determined. Although concentrations of methanol up to 100 times more than amounts initially detected in crude extract samples after freeze-drying had minimal adverse effect on the L3i, refreeze-drying of the crude extracts (2 × 24 h) further reduced methanol content to lower quantities. Thus, any

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possible larval inactivation effect by methanol was nullified.

Strain-related heterogeneity of the parasite was minimized by the establishment and maintenance of infections in two mongrel dogs from the same litter using *S. stercoralis* L3i that were obtained from a single human host. Experimental studies by Grove (1984) have shown variations in infectivity and duration of infection by different strains of *S. stercoralis* in mongrels. Some dogs were resistant to re-infection, while in others the self-cure phenomenon was absent and the infection became persistent. The likelihood of variations in the rates of larval inactivation occurring as a result of the plants' intrinsic properties was optimized. Mongrel dogs have been used as models of human strongyloidosis for more than 60 years (Sandground, 1928; Grove and Northern, 1982; Genta, 1989).

The petrol and dichloromethane crude extracts of *E. foetidum* were not miscible with LNS because of their low solubility in the aqueous medium. Thus, Triton X-100 (10 mg mL^{-1}), which is an emulsifier (Matthews, 2000), was used to bring about a homogeneous and stable dispersion of the extracts in the incubation solution. It was also the lowest concentration investigated and had a negligible adverse effect on the L3i. Also, the absence of triolein and stigmasterol from the *E. foetidum* petrol crude extract was noted because both compounds commonly occur in a wide variety of plants and possess insecticidal activity (Ito *et al.* 1964; Chippendale and Mann, 1972). This hinted at the possibility of a novel anthelmintic *E. foetidum* compound or group of compounds.

Infrared, NMR and mass spectroscopic analysis revealed eryngial to be an intensely odorous, herbaceous, volatile, non-polar and lipophilic unsaturated, long-chain fatty aldehyde. The compound's lipophilicity may partially explain its rapid rate of larval inactivation, possibly as a result of ease of diffusion across the cuticle. Many nematicides, for example the organophosphates, alkyl halides and oxime carbamates, owe their efficacy, in part, to being lipophilic (Lee and Atkinson, 1965). Eryngial's 'fast-acting' activity, based on 24 and 48 h LD_{50} values, compared favourably with ivermectin *in vitro* and underscores its candidacy for drug development studies.

A comparison can be made between the results of this and an *in vitro* study by Boonmars *et al.* 2005. In that study, aqueous and alcohol extracts (2 mg mL^{-1}) of the medicinal plant *Cardiospermum halicacabum* immobilized *S. stercoralis* L3i within 48–72 h, while ivermectin (0·25 mg mL^{-1}) immobilized the larvae between 72 and 144 h. While the authors in this previous study did not conclusively demonstrate larval mortality or determined LD_{50} values of compounds tested, ivermectin (0·25 mg mL^{-1}) taking greater than 72 h to immobilize the L3i, points in the direction of results obtained in the present study. Here, based on the 24 and 48 h LD_{50} values (1·97 mg mL^{-1} = 2·251 mM and 0·437 mg mL^{-1} = 0·499 mM, respectively), higher concentrations of ivermectin were required to effect larval inactivation in shorter durations.

The mode of action of eryngial against *S. stercoralis* is not known. However, paralysis in nematodes and other animals by drugs is primarily due to a block of neuromuscular transmitters (Holden-Dye and Walker, 2007). Such drugs include pyrantel, levamisole, santonin, bephenium salts and methyridine (Lee and Atkinson, 1965; Gustafsson *et al.* 1987; Atchison *et al.* 1992). Some anthelmintics exert activity by disrupting the nematodes' energy-producing metabolic pathways (Lee and Atkinson, 1965). Thus, mode of action studies to investigate eryngial's anthelmintic potential is timely.

Recent studies on eryngial highlight other properties and benefits of the compound. While *E. foetidum* has wide-ranging medicinal properties (Paul *et al.* 2011), its unsaturated aldehyde, eryngial, in particular, belongs to a class of compounds that are associated with inactivation of rabbit liver cytochrome P450 isoforms (Raner *et al.* 1997); is one of the defensive secretions of the spiroboloid millipede *Rhinocricus insulatus* (Wheeler *et al.* 1964); and has antibacterial activity (Kubo *et al.* 2004; Forbes and Steglich, 2010). Eryngial is also closely related to *cis*-7-dodecenal, which possesses sex pheromone activity (Chisholm *et al.* 1982), and may thus be further investigated for insecticidal activity against some moth species, for example, *Malacosoma disstria* (Hübner).

Eryngial, which is also known for its application as a food flavouring (Rowe, 1998), was listed in 2000 by the Japanese Flavour and Fragrance Material Association as an often-used flavouring agent in its food industry. This is not surprising, as *E. foetidum* is widely used throughout the Caribbean, Latin America and the Far East as a food flavouring and seasoning herb for meat and other foods (Ramcharan, 1999; Chhetri, 2006; Kar and Borthakur, 2007; Prasad *et al.* 2008). The usage of eryngial in the food industry suggests that it is safe for use in humans.

The results of this study indicate that eryngial has tremendous potential as a compound of medicinal value, and is a prime candidate for further drug development studies, including mammalian toxicity. Eryngial's simple structure coupled with its acute toxicity against *S. stercoralis* infective larvae *in vitro* would favour synthesis in the laboratory, and its development into a potentially safe and inexpensive drug for pharmacological application, especially in endemic regions. Superior efficacy of such an anthelmintic drug would be most likely due to its ability to arrest or lessen the rate of autoinfection by the L3i, the phenomenon that plays an important role in maintaining *S. stercoralis* infections in infected

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individuals. Inactivation of large numbers of infective larvae and, potentially, adult worms in the intestine would be expected to result in reduced risk of morbidity. Furthermore, based on comparable activity to ivermectin, eryngial should be scrutinized for mammalian toxicity and its potential broad-spectrum anthelmintic activity *in vitro* and *in vivo*. Eryngial may thus not only be further investigated for development into a phytopharmaceutical anthelmintic against strongyloidosis and other nematode infections, but also for its use as a microbicide and an alternative, ecologically safe insect pest control agent.

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