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Anthelmintic properties of traditional African and Caribbean medicinal plants: identification of extracts with potent activity against *Ascaris suum* in vitro

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Abstract – Ascariasis affects more than 1 billion people worldwide, mainly in developing countries, causing substantial morbidity. Current treatments for *Ascaris* infection are based on mass drug administration (MDA) with synthetic anthelmintic drugs such as albendazole, however continual re-infection and the threat of drug resistance mean that complementary treatment options would be highly valuable. Here, we screened ethanolic extracts from 29 medicinal plants used in Africa (Ghana) and the Caribbean (US Virgin Islands) for in vitro anthelmintic properties against *Ascaris suum*, a swine parasite that is very closely related to the human *A. lumbricoides*. A wide variety of activities were seen in the extracts, from negligible to potent. Extracts from *Clausena anisata*, *Zanthoxylum zanthoxyloides* and *Punica granatum* were identified as the most potent with EC 50 values of 74, 97 and 164 µg/mL, respectively. Our results encourage further investigation of their use as complementary treatment options for ascariasis, alongside MDA.

Key words: *Ascaris suum*, Anthelmintic, *Clausena anisata*, *Zanthoxylum zanthoxyloides*, *Punica granatum*.

Introduction

Soil-transmitted helminths remain one of the largest burdens on global health. Altogether, *Ascaris lumbricoides* (roundworm), *Trichuris trichiura* (whipworm) and *Necator americanus* (hookworm) infect more than a billion people, mainly in the developing world [20, 24]. Of these, the most prevalent is *A. lumbricoides*, which was estimated to infect around 800 million people in 2010, resulting in more than a million years lived with disability (YLD) [20]. Ascariasis can result in malnutrition and inhibit cognitive development and learning in children, and can also interfere with effective expression of immunity to other pathogens and vaccines due to polarisation of the immune system towards a regulatory/Th2-skewed state [6, 21].

At present, *A. lumbricoides* infections are treated through mass drug administration (MDA) programmes, involving annual or bi-annual treatment of school children, mainly with...
albendazole [12]. However, the sustainability of this approach has been questioned due to continual re-infection, arising from the hard-shelled eggs which survive for many years in the environment, and the ever-present threat of drug resistance, where cautionary tales can be drawn from the critical levels of resistance that have arisen in helminths of veterinary importance due to mass administration of synthetic drugs [11, 13, 23].

The use of indigenous medicinal plants for control of internal parasites has been practised for centuries, however scientific validation of these traditional practices has been lacking [8, 22]. Thus, a potentially vast resource of natural plant compounds with anthelmintic activity has been underutilised. The use of natural plant extracts, which can be prepared by decoc- tion or other simple procedures, has several advantages such as low cost and easy integration into local community practices, if and where plants are locally available. The rational use of such an approach may complement current MDA programmes and be a useful tool to slow the onset of drug resistance.

Screening for anthelmintic activity is often accomplished by using the free-living nematode Caenorhabditis elegans [5, 9] due to low cost and amenability of laboratory culture methods. However, many differences exist between free-living nematodes and parasites such as Ascaris, as well as between free-living and parasitic stages of the same species [17]. Thus, anthelmintic activity is ideally assessed using as close as possible a model to the eventual target. Ascaris suum is a swine parasite and closely related parasite to A. lumbricoides, being morphologically indistinguishable, and indeed, for many years the human and pig forms of Ascaris were considered to be a single species [16]. This means that A. suum is an excellent model for studying possible new interventions such as vaccines or new drug candidates against A. lumbricoides. In particular, in vitro studies with A. suum are easily performed due to the ability to generate large numbers of infective, parasitic third-stage larvae (L3) from embryonated eggs recovered from the uteri of adult female worms. We have previously used a combination of motility and migration inhibition assays to test for in vitro anthelmintic effects of a number of compounds against A. suum L3 [25]. Here, we utilised local ethno-medical knowledge to compile a library of traditional medicinal plants from Ghana and the Caribbean, and used A. suum L3 to screen > 30 extracts for in vitro activity. Our results indicate that some of these plants may have value as a complementary treatment option for ascariasis.

Materials and methods
Plant material and extraction
Medicinal plants were collected during the period November 2013 to January 2014 in the Greater Accra region of Ghana or October to November 2014 on the US Virgin Islands (Table 1). The plants were dried at ambient temperature in the shade. The plants were identified and authenticated by an ethnobotanist (Jens Soelberg, University of Copenhagen, Denmark). Botanical voucher specimens were deposited at the Herbaria at the University of Copenhagen, Denmark; and at the University of Ghana, Ghana, and St. George Estate Botanical Garden, respectively. Voucher numbers are given in Table 1. Ethanolic extracts were prepared by extracting 2 g of ground plant material with 20 mL of 96% ethanol in an ultrasonic bath for 30 min. Extracts were filtered through filter paper and taken to dryness at room temperature under nitrogen. For anthelmintic assays, extracts were dissolved in 100% DMSO.

Parasite material
Gravid Ascaris suum worms were collected from fresh pig intestines at a local slaughterhouse (Danish Crown, Ringsted, Denmark). Eggs were isolated from the uteri of the worms and then subsequently embryonated at 25 °C for at least 60 days in 0.1 M H2SO4. Full embryonation was confirmed by light microscopy. To obtain the L3, eggs were washed, suspended in Hanks’ Buffered Salt Solution (HBSS) and then hatched by stirring together with 2 mm glass beads for 30 min at 37 °C. Viable L3 were then separated from unhatched eggs and debris by overnight migration into sterile HBSS using a Baermann apparatus equipped with 20 µm mesh. The L3 were then washed, counted and suspended in larval culture media (RPMI 1640 supplemented with 2 mM L-glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin) for use in the migration inhibition assay.

Larval migration inhibition assay
The migration inhibition assay was conducted essentially as described previously [25]. Briefly, 100 L3 (in triplicate) were added to wells on a tissue culture plate and plant extracts in DMSO added (DMSO concentration never exceeded 1%). All assays included 1% DMSO in culture media as a negative control and 50 µg/mL ivermectin (Sigma-Aldrich) as a positive control. The plates were then incubated overnight at 37 °C in 5% CO2 in air. Then, an equal amount of 1.6% agar solution (45 °C) was added to each well and mixed thoroughly. The agar was allowed to solidify, before fresh culture media were added on top to cover the agar and the plates returned to the incubator overnight. The next day, the media were collected from each well and the number of larvae that had migrated from the setting agar was enumerated by light microscopy.

Data analysis and statistics
Migration inhibition was calculated relative to larvae incubated only in culture media (+1% DMSO) and expressed as percentage inhibition. Half-maximal effective concentration (EC50) values were calculated using non-linear regression. Analyses were performed in GraphPad Prism (v6.00, GraphPad Software, La Jolla, California, USA, www.graphpad.com).

Results and discussion
Screening of the plant extracts at a concentration of 1 mg/mL revealed a wide variety of potencies (Fig. 1).
Table 1. Names and characteristics of plant species tested for anthelmintic activity against *Ascaris suum*. Plants were collected from either Ghana (G) or the US Virgin Islands (VI).

| Species name                                      | Location | Family             | Voucher | Plant part      |
|---------------------------------------------------|----------|--------------------|---------|-----------------|
| *Aloe vera* (L.) Burm. f.                         | VI       | Xanthorrhoeaceae   | JS 617  | Leaves          |
| *Boerhavia diffusa* L.                           | G        | Nyctaginaceae      | JS 281  | Aerial parts    |
| *Boerhavia erecta* L.                            | G        | Nyctaginaceae      | JS 282  | Aerial parts    |
| *Clausena anisata* (Willd.) Hook. ex Benth.       | G        | Rutaceae           | JS 214  | Roots           |
| *Deinbollia pinnata* Schum. & Thonn.              | G        | Sapindaceae        | JS 202  | Aerial parts    |
| *Erythrina senegaletensis* DC.                    | G        | Logiaceae          | JS 231  | Bark            |
| *Flacourtia flavescens* Wild.                     | G        | Salicaceae         | JS 249  | Leaves          |
| *Flueggea virosa* (Roxb. ex Willd.) Royle        | G        | Phyllanthaceae     | JS 252  | Leaves          |
| *Gardenia ternifolia* Schumach. & Thonn.         | G        | Rubiaceae          | JS 246  | Leaves          |
| *Gynnanthemum coloratum* (Willd.) H. Rob. & B. Kahn| G        | Asteraceae         | JS 268  | Leaves/flowers  |
| *Gynnanthemum coloratum* (Willd.) H. Rob. & B. Kahn| G        | Compositae         | JS 268  | Roots           |
| *Launaea taraxacifolia* (Willd.) Amin ex C. Jeffrey| G        | Compositae         | JS 212  | Leaves          |
| *Mallotus oppositifolius* (Geisele) Müll. Arg.   | G        | Euphorbiaceae      | JS 208  | Leaves          |
| *Mucuna pruriens* (L.) DC.                       | VI       | Leguminosae        | JS 656  | Fruit           |
| *Newbouldia laevis* (P. Beauv.) Seem.             | G        | Bignoniaceae       | JS 216  | Leaves          |
| *Opuntia* sp.                                     | VI       | Cactaceae          | JS 672  | Stem            |
| *Paullinia africana* R.Br. ex Tedlie               | G        | Sapindaceae        | JS 219  | Aerial parts    |
| *Phyllanthus amarus* Schumach. & Thonn.           | G        | Phyllanthaceae     | JS 237  | Aerial parts    |
| *Premna quadrifolia* Schumach. & Thonn.           | G        | Lamiaceae          | JS 283  | Aerial parts    |
| *Psidium guajava* L.                              | VI       | Myrtaceae          | JS 623  | Leaves          |
| *Punica granatum* L.                              | VI       | Lythraceae         | JS 615  | Fruit peel      |
| *Pupalia lappacea* (L.) Juss.                     | G        | Amaranthaceae      | JS 239  | Aerial parts    |
| *Rivina humilis* L.                               | VI       | Phytolaccaceae     | JS 608  | Aerial parts    |
| *Senna occidentalis* (L.) Link                    | G        | Leguminosae        | JS 234  | Aerial parts    |
| *Senna occidentalis* (L.) Link                    | G        | Leguminosae        | JS 234  | Roots           |
| *Spathodea campanulata* P. Beauv.                 | G        | Bignoniaceae       | JS 230  | Bark            |
| *Spigelia anethmia* L.                            | VI       | Logiaceae          | JS 651  | Aerial parts    |
| *Stylosanthes erecta* P. Beauv.                   | G        | Leguminosae        | JS 271  | Aerial parts    |
| *Thorningia sanguinea* Vahl                       | G        | Balanophora        | JS 296  | Aerial parts    |
| *Triumfetta semitriloba* Jacq.                    | VI       | Malvaceae          | JS 658  | Aerial parts    |
| *Zanthoxylum zanthoxyloides* (Lam.) Zepern. & Timler| G        | Rutaceae           | JS 243  | Roots           |
| *Zanthoxylum zanthoxyloides* (Lam.) Zepern. & Timler| G        | Rutaceae           | JS 243  | Root bark       |

A number of extracts had no or negligible activity. However, 10 extracts inhibited larval migration by at least 50%. An arbitrary cut-off value of 90% migration inhibition was chosen to define plants that had potent activity, comparable to the inhibition achieved by 50 µg/mL ivermectin (positive control). Using this criterion, four extracts – *Clausena anisata*, *Zanthoxylum zanthoxyloides* (both the roots and root bark) and *Punica granatum* – were selected on the basis of potent activities for dose-dependent studies to confirm their activities and determine EC_{50} values. All four extracts displayed dose-dependent activity (Fig. 2). The EC_{50} (95% CI) values for *C. anisata*, *Z. zanthoxyloides* roots, *Z. zanthoxyloides* root bark and *P. granatum* were 74 (63.3–86.8), 97 (63.9–149.3), 132 (105.9–164.6) and 164 (124.7–271.1) µg/mL, respectively.

We have thus confirmed that a number of plants that are traditionally used in medicinal form in *Ascaris*-endemic regions have direct anthelmintic activity against *A. suum*. The identification of three extracts with particularly potent activity is consistent with previous reports concerning their ethno-medical usage and anthelmintic properties. *C. anisata* is used as an anthelmintic by traditional healers in Kenya [24], and has also been noted to have *in vitro* activity against free-living larvae of the sheep nematode *Haemonchus contortus* [23]. Similarly, *in vitro* studies with *Z. zanthoxyloides* have demonstrated anthelmintic effects of crude extracts from this plant against *H. contortus* and another sheep nematode, *Trichostrongylus colubriformis* [4, 10]. Moreover, the distilled essential oils from *Z. zanthoxyloides* have been shown to have *in vitro* activity against the rat helminth *Strongyloides ratti* [18]. *Punica granatum* extracts also have *in vitro* activity against free-living worms such as *Allolobophora caliginosa* [7] and veterinary helminths such as the poultry roundworm *Ascaridia galli* [3]. Our data, which indicate a strong anti-*Ascaris* effect of these three plant extracts, extend on these previous studies by suggesting that they may have potential for treatment of *A. lumbricoides* in humans.

The active compounds of these extracts were not investigated here, but all three extracts (*Z. zanthoxyloides*, *P. granatum* and *C. anisata*) are known to be rich in secondary compounds such as tannins (with *P. granatum* being a particularly rich source of ellagitannins and gallic acid), flavonoids, terpenes and alkaloids, and these have been implicated in the apparent anti-microbial and ethno-medical properties of these plants [1, 14, 19]. Previous *in vitro* studies with *H. contortus*...
and *S. ratti* have suggested that the anthelmintic properties of *Z. zanthoxyloides* are partially dependent on flavonoids and related polymeric tannins, but that other bioactive compounds such as terpenes derived from the essential oil may contribute to the activity [4, 18]. Furthermore, a related plant, *Z. liebmannianum*, has also been shown to have activity against *A. suum in vitro* and here *α*-sanshool was isolated as a putative active compound [15]. Ellagitannins or related phenolic compounds found in *P. granatum* have been speculated to be responsible for activity that has been observed against protozoan parasites such as *Cryptosporidium parvum* [2]. Fractionation and identification of the active compounds in these extracts is ongoing in our laboratory.

The confirmation of anti-*Ascaris* activity of some of these traditional extracts encourages refinement of their use as treatments for roundworm infection in areas where these plants are found locally. Whilst our current experiments were performed with third-stage larvae, which are present in the intestinal tract for only a short period of time following egg ingestion, we have shown previously that activity of bioactive compounds against *L3* correlates very closely with their activity against both fourth-stage larvae [25] and adult worms (A.R. Williams, unpublished data). Thus, there appears to be clear scope for these remedies to be used as therapeutic treatments against established infections in the small intestine. Having now confirmed the activity of these traditional extracts, further studies to investigate the pharmacokinetics in order to determine the best possible dosage and administration methods of these

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**Figure 1.** Inhibition of migratory ability of *Ascaris suum* third-stage larvae after exposure to extracts of medicinal plants (1 mg/mL) or ivermectin (50 μg/mL). Results are the mean (± SEM) of three replicates from a single experiment. The vertical dashed line indicates 90% inhibition of larval migration.

**Figure 2.** Dose-dependent inhibition of migration of *Ascaris suum* third-stage larvae after exposure to extracts of three different medicinal plants. Results are the mean of three independent experiments, each performed in triplicate, with the results expressed as the mean ± inter-experiment SEM.
extracts are now warranted. Moreover, if activity can be solely or mainly ascribed to a single compound, then the possibility remains of synthesis and production of a new anthelmintic drug.

In conclusion, we have demonstrated that extracts of *C. anisata*, *Z. zanthoxyloides* and *P. granatum* have potent anthelmintic activity against *A. suum* in vitro, which encourages further investigation of their use as therapeutic agents against *Ascaris* infections in endemic regions.

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