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Ascaridole-less infusions of *Chenopodium ambrosioides* contain a nematocide(s) that is(are) not toxic to mammalian smooth muscle

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Abstract

Infusions of *Chenopodium ambrosioides* (L.) have been used for centuries in the Americas as a popular remedy against intestinal worm infections. The essential oil of *Chenopodium ambrosioides* contains high levels of ascaridole, which is a potent anthelmintic, but which has also been responsible for human fatalities, leading to its disuse. Almost 90% of the nematocidal activity of *Chenopodium ambrosioides* infusions was due to a hydrophilic component different from ascaridole. Synthetic ascaridole and the ascaridole from infusions, extracted into hexane, caused a reduction of carbachol-induced contractions in rat gastrointestinal smooth muscle at concentrations required to kill *Caenorhabditis elegans* (L.). The herbal infusion and the ascaridole-free hexane-extracted aqueous residue of the above infusion, at nematocidal concentrations, had no detectable effect on smooth muscle contraction in the above system. It would appear that the traditional form of useage of *Chenopodium ambrosioides* infusions as a vermifuge is safer than the use of the herb's essential oil.
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1. Introduction

The weed *Chenopodium ambrosioides* (L.), known as Epazote in Mesoamerica and as Paico in the Andes, has been used for many centuries as an anthelmintic (Morton, 1980; Millspaugh, 1892). Beginning in the 18th century, *Chenopodium ambrosioides* was taken to the rest of the world, being widely adopted as an anthelmintic remedy (Kliks, 1985).

The traditional way to prepare Epazote in Central America is to make an infusion of the dry plant in boiling water. This preparation is ingested as soon as it cools.

Beginning in the 19th century, *Chenopodium ambrosioides* was steam distilled to produce *Chenopodium* oil, a potent anthelmintic. This became an industry and by the 1900's "Baltimore oil", as it became known, was widely used to rid patients of worms.

Smillie and Pessoa (1924) were the first to show that the anthelmintic properties of *Chenopodium* oil were due to the compound ascaridole, which constituted more than 50% of the weight of the oil (Nelson, 1920; Paget, 1926; Johnson and Croteau, 1984).

Although Kliks (1985) could find no record of poisoning or fatalities arising from the ingestion of Epazote infusion, there were numerous reports of both resulting from the intake of *Chenopodium* oil (Levy, 1914). Fatalities arising from the consumption of *Chenopodium* oil were probably due, in most cases, to overdoses (Levy, 1914; Paget, 1926) yet they led to the commercial decline of the use of the oil, in favour of more modern medications.

Salant and Mitchell (1916, 1917) found that *Chenopodium* oil first increased and eventually abolished contractions of the duodenum of rabbits, cats and dogs and led to decreased contractions of isolated frog heart. When injected into the blood stream of animals, the oil led to a depression of circulation, respiration and intestinal movements. Okuyama et al. (1993) found that ascaridole, when given to mice at a dose of 100 mg/kg body weight produced hypothermia, and decreased locomotory activ-

ity. When given at triple the above dose, the animals died.

Pollack et al. (1990) observed that ascaridole inhibited the growth of *Plasmodium falciparum* in vitro, and that cineol, which is a very similar compound lacking the internal 1,4 peroxide was inactive. Artemisinin, on the other hand, which like ascaridole contains a 1,4 endoperoxide, was active against *Plasmodium falciparum*. These observations prompted Pollack et al. (1990) to conclude that the endoperoxide in ascaridole was essential for its anthelmintic activity.

In this communication we demonstrate that, whereas ascaridole, at a concentration that immobilised or killed *Caenorhabditis elegans*, had a profound effect on rat gastrointestinal smooth muscle contractions, infusions of *Chenopodium ambrosioides* and ascaridole-free infusions of the weed had no such effect on the above smooth muscle preparations, at concentrations that effectively immobilised and killed *Caenorhabditis elegans* in vitro.

2. Materials and methods

2.1. Strains and materials

Caenorhabditis elegans (L.) strain N2 was obtained from Dr. J. Culotti, Hospital for Sick Children, Toronto, Ont., Canada.

Epazote *Chenopodium ambrosioides* (L.) family, Chenopodiaceae (Wiersma and Leon, 1999) seeds were obtained from Richter's Herbs, Goodwood, Ont. L0C 1A0, Canada (http://www.richters.com) Seeds were planted in potting soil and grown in the greenhouse under a 16 h light and 8 h dark regime and harvested when about 0.5 m tall. The green part of the plant was then air dried on paper towels at 25 °C for 7–10 days and stored in the dark in Styrofoam boxes at room temperature.

2.2. Growth and maintenance of Caenorhabditis elegans and toxicity assay

Caenorhabditis elegans was maintained on solid NG medium (Sulston and Hodgkin, 1988). Between 20 and 50 nematodes were placed in 500 µl of NG medium (Sulston and Hodgkin, 1988) in microtitre wells (Falcon No 3047, Becton Dickinson) containing 30 µl of five times concentrated overnight culture of *Escherichia coli* WP2 (*uvr A trp malB,* Witkin, 1975) maintained in Luria Broth (Berman et al., 1982) and resuspended in NG medium. Aliquots (5–200 µl) of aqueous extracts (in water or 10 mM potassium phosphate, pH 6) were added directly to the wells, while concentrated oils, and chemically synthesised Ascaridol were diluted with dimethyl sulfoxide (DMSO) prior to being added to the wells in 7.5 µl volumes. Dilute extracts, prepared in, or extracted into non-aqueous solvents, were concentrated first by evaporation under N_2, the solvent-free residue was then resuspended in 7.5 µl of DMSO, which

was then added to the test wells. Controls with the equivalent volumes of DMSO alone were not noticeably affected. Nematode survival was measured 4–5 h after addition of the extracts and again 12–13 h later.

One LC_{90} unit of activity is defined as that quantity of preparation that kills (or permanently immobilizes) >90% but <100% of the nematodes in the above assay within 12 h.

2.3. Preparation of extracts

Dried Epazote, containing leaves, fruit and flowers, was blended in a Waring blender for 10 min in 10–15 volumes (v/w) of boiling water that had just been removed from a heating block. The mash was then filtered through MIRACLOTH (Calbiochem, San Diego, California), and the filtrate centrifuged at 27,000 g for 20 min at 4 °C in a Sorvall Refrigerated Centrifuge. The supernatant was used directly (crude extract), or extracted three times with an equal volume of hexane, giving rise to a hexane layer (containing ascaridole) and an aqueous layer (aqueous residue). The hexane layer was evaporated under partial vacuum at 40–50 °C and the resulting oil either used directly, or diluted in DMS0 immediately before use, or stored at −20 °C. The aqueous layer was subjected to evacuation at room temperature for 2.5 h prior to use, or stored at 0–4 °C for 1–2 days before doing so.

2.4. Synthesis of ascaridole

Ascaridole was prepared from α-terpinene, following the procedure of Aubry and Bouttemy (1997) and purified by distillation under partial vacuum. Purity was greater than 95%, as judged by NMR spectroscopy and the yield was approximately 35%.

2.5. Physiological experiments

Female adult Sprague–Dawley rats were used in all experiments. Rats were killed by cervical dislocation. The abdomen was opened by midline incision and the tissues being used (either stomach, fundus, jejunum or ileum) were excised. Tissues were immediately placed in a physiological Krebs solution at room temperature consisting of (in mM): NaC1, 116; KC1, 4.6; $MgC1_2$, 1.2; $CaCl_2$, 1.5; NaH_2PO_4, 1.2; $NaHCO_3$, 22; glucose, 0. For experiments with the jejunum and ileum the intestine was cut, using scissors, along the mesenteric line and cleaned of all intraluminal contents in Krebs solution. The tissues were pinned and gently stretched in a Petri-dish containing Krebs solution and bubbled with 95% O_2–5% CO_2. Strips approximately 1 cm in length were divided down the middle to give a matching pair of tissues about 10 mm long and 3 mm wide. For experiments using the gastric fundus, the stomach was opened and cleaned of all contents in Krebs solution. Fundus strips approximately 10 mm long and 3 mm wide were removed from the stomach, with all work done in a Petri dish containing

Krebs solution, and bubbled with 95% O_2–5% CO_2. Tissues from all areas of the gastrointestinal tract being investigated were tied at both ends with surgical silk. Using the silk, one end of the tissue was anchored at the bottom of a 5 ml organ bath and the other end was attached to a Grass Model 7D force transducer that was coupled to a pen recorder for isometric tension recording. The strips were suspended under 1 g of tension as described by Aube et al. (1996) for rat intestine segments. The 5 ml organ baths contained Krebs solution that was continuously bubbled with 95% O_2–5% CO_2 and were maintained at 37 °C by a warm water jacket.

Tissues were allowed to equilibrate under tension for 30–45 min prior to the addition of drugs. Addition of carbachol elicited a response that remained steady till the drug was washed out. When responses had stabilised, tissues were washed and allowed to recover for another 30 min prior to a second addition of the agonist. Once two stable responses had been obtained, the effects of ascaridole were tested. The drug was added 5 min prior to the agonist. To assess recovery, tissues were washed out with Krebs solution and a final response to carbachol elicited. A similar protocol was followed in testing the infusion, the ascaridole-containing hexane layer or the ascaridole-free aqueous layer of a hexane-extracted infusion, or a solution of potassium.

2.6. Data analysis

All data are presented as means plus or minus the standard error of the mean significance among groups was tested using unpaired Student's *t*-tests.

3. Results

3.1. Most of the nematocidal activity in aqueous infusions of Chenopodium ambrosioides is not due to ascaridole

Ascaridole is very poorly soluble in water (Merck Index, 1989) and can be extracted out of an aqueous solution and into the hexane layer by shaking it with hexane. When a supersaturated solution of water containing ascaridole, synthesised by the procedure of Aubry and Bouttemy (1997), was shaken three times with an equal volume of hexane, all the measurable nematocidal activity was found in the hexane layer and none remained in the aqueous one (results not shown).

When an aqueous infusion of *Chenopodium ambrosioides* was shaken with hexane as described above, close to 90% of the nematocidal activity remained in the aqueous residue (Table 1) and the remainder was found in the hexane layer. When subsequent to this, the aqueous residue was shaken six more times, each time with an equal volume of hexane, the aqueous residue retained all its bioactivity and the additional hexane layers contained none (results not shown).

Table 1
Nematode killing activity in fractionated Epazote infusions

Fraction	Yield of worm killing activity in fraction (%) average ± S.D. (*N*)[b]	LC$_{90}$ units/µl average ± S.D. (*N*)
Crude infusion	100 (24)	0.016 ± 0.009 (29)
Aqueous layer[a]	88 ± 15 (24)	0.013 ± 0.007 (28)
Hexane layer oil[a]	7 ± 7 (22)	4 ± 2 (8)
Synthetic ascaridole	–	11 ± 3 (17)

The crude infusions and aqueous residues were from different preparations. The bioactivity of crude infusions was set as 100%. The aqueous layers were the ascaridole-free aqueous layers, resulting from extraction of the infusions with hexane. The eight hexane layer solvent-free oils tested were from eight separate preparations in which the hexane layer, resulting from hexane extraction of the infusion was evaporated to a hexane-free oil. The synthetic ascaridole was tested over a period of 1 year.

[a] From hexane extraction of infusion.

[b] *N*: number of experiments.

Ascaridole, synthesised from α-terpinene by the procedure of Aubry and Bouttemy (1997) contained between 7 and 14 LC$_{90}$ units of nematocidal activity per microlitre and was 95% pure, as judged by the NMR spectrum (results not shown), the 5% impurity was α-terpinene, which was inactive in our bioassay (see the following).

One of the hexane layers of *Chenopodium ambrosioides* infusion, extracted thrice with hexane was evaporated, to remove the hexane, and the resulting oil contained 13 LC$_{90}$ units of nematocidal activity per microlitre. The NMR spectrum of the above oil (Fig. 1) indicates that ascaridole comprised more than 90% of the material in it. The infusions were variable in terms of their potency, as were the different fractions prepared from them (Table 1). However, it is clear that when the infusions were extracted with hexane, to remove the ascaridole, most of the nematocidal activity was found in the aqueous layers which have no ascaridole in them. The hexane layers contained all the ascaridole in the infusion, which accounted for about 10% of the bioactivity; a deep yellow waxy compound that is not ascaridole was also found in the hexane layer.

Johnson and Croteau (1984) found that the oil of *Chenopodium ambrosioides* contained between 16 and 61% ascaridole, 22–77% hydrocarbons, 2–10% *p*-cymene, 7–53% α-terpinene, 0.3–0.7% γ-terpinene, 5–48% limonene, 1–8% *iso*-ascaridole, 0.2–1% *trans*-diol and 0.1–0.4% *cis*-diol derived from ascaridole. One microlitre of *p*-cymene, α-terpinene, γ-terpinene, limonene and ascaridole reduced by the procedures of Johnson and Croteau (1984) to produce separately the *cis*- and *trans*-diols, were all inactive in our nematocidal assay.

It would appear from the above that when *Chenopodium ambrosoides* infusions are extracted with hexane, all the ascaridole is extracted into the hexane layer and that the aqueous layer retains most of the nematocidal activity. Consequently, it would appear, that most of the nematocidal activity in the infusion and all of the nematocidal activity in the hexane-extracted aqueous layer, is not ascaridole.
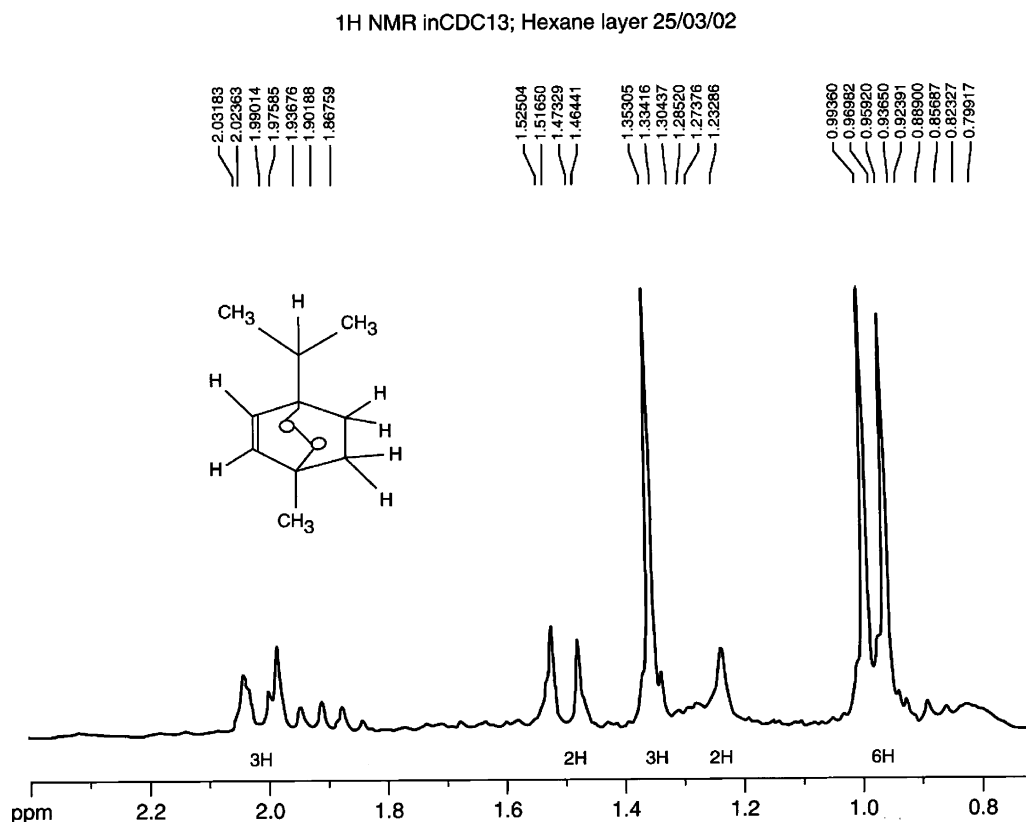
Fig. 1. NMR spectrum of hexane layer from hexane-extracted Epazote infusion.

The above results, by themselves, would not completely rule out the possibility that ascaridole is being synthesised from its precursors by enzymes in the aqueous layer (Johnson and Croteau, 1984). This possibility was tested directly, by passing the infusion through an Amicon Ultra-filtration membrane (Millipore Corp., Bedford, MA 01730, USA)—the iodoperoxidase that catalyzes ascaridole synthesis has a molecular weight above 40 kDa according to Johnson and Croteau (1984)—and subsequently carrying out the hexane extraction on the Filtrate. The same result was obtained as reported above (results not shown): more than 80% of the nematocidal activity was found in the aqueous layer. Conversely, a 1 ml sample of the aqueous layer, resulting from hexane extraction, was dialysed against 1 l of water and the sample lost all its nematocidal activity (results not shown), indicating that the nematocidal activity is not a macromolecule.

3.2. Ascaridole at nematocidal concentrations reduces basal tone, and decreases phasic contractions as well as carbachol-induced contractions of rat gastrointestinal tissue in vitro

The *Chenopodium ambrosoides* infusion and the ascaridole-free infusion, at nematocidal concentrations, did none of the above. The results in Fig. 2 show that carbachol (10^{-6} M) elicited a rapid phasic and a sustained

tonic contraction in the rat fundus. Removal of the agonist leads to a rapid cessation of the response. The addition of 5.4×10^{-3} M ascaridole (a nematocidal concentration), decreased the basal tone of the rat fundus and markedly inhibited the response to a subsequent addition of carbachol (10^{-6} M). Washing out of the drug leads to complete recovery of the response. Similar results were seen in other tissues such as the ileum and the jejunum.

The results suggested that ascaridole had clear inhibitory effects on the gut. Experiments were done to assess the effects of the *Chenopodium ambrosoides* infusion itself as well as the infusions from which the ascaridole had been removed by hexane extraction. Both had nematocidal activity. The data shown in Fig. 3 indicate that neither the infusion nor the ascaridole-free infusion had any significant inhibitory effect, at nematocidal concentrations, on carbachol-induced contractions. By contrast chemically synthesised ascaridole clearly did. We also tested the effect of the hexane layer of the hexane-extracted infusion. This would have contained all the ascaridole originally present in the infusion. As shown in Fig. 3, this hexane layer also inhibited carbachol-induced responses. Another important control was needed. Plant extracts contain significant amounts of K^+ and this itself could have effects on smooth muscle. Assays showed that the infusions contained 11 mM K^+, but the addition of this amount of K^+ had no inhibitory effect on carbachol-induced contractions of smooth muscle (results not shown).
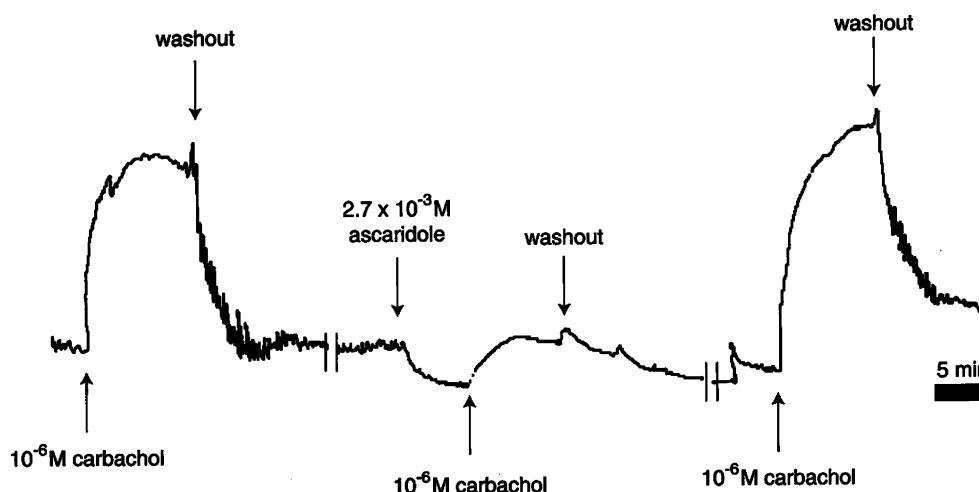
Fig. 2. Effect of 2.7×10^{-3} M ascaridole on contractions in the rat gastric fundus induced by 10^{-6} M carbachol. All drugs were added directly to the bath solution. Ascaridole was added to the bath 5 min prior to the application of carbachol. Washout was followed by a 30-min recovery period.
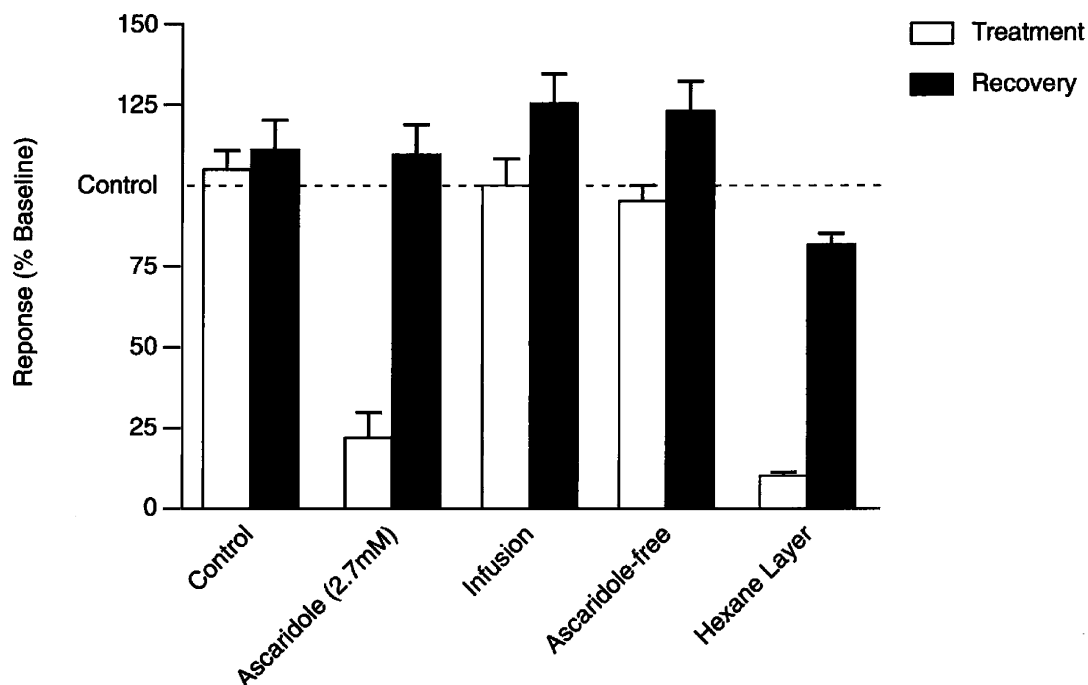

Fig. 3. Effect of various additions to organ bath mixture on carbachol-induced contractions in rat gastric fundus. Control, $n = 5$ from four animals; ascaridole, 2.7×10^{-3} M ascaridole added in 20 μl DMSO to 5 ml organ bath, $n = 7$ from seven animals; infusion, 700 μl infusion was added to 4.20 ml Krebs, $n = 10$ from three animals; ascaridole-free infusions, resulting from hexane extractions of the infusions, 800 μl were added to 4.2 ml Krebs solution, $n = 9$ from four animals; ascaridole-containing hexane layer, resulting from hexane extractions of infusions, 75 ml of the hexane layer from these extractions were evaporated to remove the hexane and the remaining oil reconstituted in 20 μl DMSO, which was then added to bath solutions, $n = 2$ from one animal. All concentrations of additions were at levels that permanently immobilised *Caenorhabditis elegans* oil. The effects of the hexane layer and ascaridole are both significantly different from the controls.

4. Discussion

The use of *Chenopodium* oil as an anthelmintic for humans has been discontinued, in favour of more "modern" medicines, primarily because of its toxicity (Paget, 1926). Indeed there have been several reports of poisoning and even fatalities of humans caused by the above oil (Paget, 1926; Levy, 1914; Montoya-Cabrera et al., 1996).

Chenopodium oil has been shown to cause depression of movement and eventual paralysis of mammalian gastrointestinal tissue in vitro and in vivo (Salant and Mitchell, 1916). More recently, methanolic extracts of Epazote

(*Chenopodium ambrosioides*) which would be expected to contain ascaridole, have been found to exert spasmolytic activity on acetylcholine-contracted rat ileum (Garcia et al., 1997).

Our results confirm the above observations, showing that both synthetic ascaridole and the ascaridole extracted into hexane from of *Chenopodium ambrosoides* infusions, relax rat gastrointestinal tissue contracted by carbachol (Figs. 2 and 3). The dose of ascaridole required to relax the above tissue was the same as that required to kill *Caenorhabditis elegans* in vitro.

Although the cases of *Chenopodium* oil leading to severe poisoning and fatalities in humans were probably mostly due to overdoses (Levy, 1914; Montoya-Cabrera et al., 1996), the therapeutic dose is uncomfortably close to the toxic dose for humans.

The scientific literature does not appear to report cases of severe poisoning or death from the ingestion of aqueous infusions of *Chenopodium ambrosoides* (Kliks, 1985). This is perhaps not surprising, given our observations that on average less than 11% of the anthelmintic activity in such infusions could be ascribed to ascaridole (Table 1); the rest of the activity appears to reside in a much more hydrophylic fraction, which did not have any effect on rat gastrointestinal tissue contractions in our experiments.

Despite the fact that aqueous infusions of Epazote/Paico have been used effectively for hundreds of years, Kliks (1985) found no evidence of its anthelmintic effectiveness in human trials. This surprising observation needs to be explored further, but what can be said with reasonable confidence, is that our observations, plus the existing literature suggest that Epazote/Paico aqueous infusions, taken at recommended doses, are unlikely to produce acute poisoning or fatalities in humans, i.e. it appears to be safer than *Chenopodium* oil.

Most users of Epazote/Paico infusions do so in great part because of its low cost, relative to more modern commercial anthelmintics. A comparison between treatments of children with Paico juice and Albendazole (a commonly used anthelmintic against Ascariasis) revealed that at the recommended doses, both were equally effective therapeutically and both had some adverse (but not fatal) effects in 23% of the cases (Lopez de Guimaraes et al., 2001). Paico juice, unlike Albendazole, was also effective in the treatment of *Hymenolepsis nana* making it a preferred treatment because of its effectiveness and cost.

Chenopodium ambrosioides continues to be used widely to treat intestinal worms and other ailments in humans, with apparent success (Quinlan et al., 2002; Franca et al., 1996). Few studies, if any, have been done to examine the long term effects of Epazote extracts of all kinds on humans, and this needs to be done, given the following findings: Epazote extracts were seen to increase the frequency of chromosomal aberrations and decrease the mitotic index of human lymphocytes in vitro (Gadano et al., 2002; Ruffa et al., 2002). These extracts would be expected to contain some ascari-

dole, which could be the causative agent. If so, making infusions of the leafy portion of Epazote would minimize the ascaridole concentration. This point needs to be explored further. The fact that Epazote extracts are used as abortifacients in New Mexico and elsewhere (Conway and Slocumb, 1979) indicates that strict adherence to recommended dosages for each kind of use should be observed.

Acknowledgements

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