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In vitro and *in vivo* studies on the bioactivity of a ginger (*Zingiber officinale*) extract towards adult schistosomes and their egg production

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Abstract

The bioactivity of an ethyl acetate extract of ginger (*Zingiber officinale*) towards *Schistosoma mansoni* adult pairs, both cultured *in vitro* and *in vivo* in laboratory mice, was investigated by monitoring worm mortality and fecundity. *In vitro*, a concentration of 200 mg l^{-1} of extract killed almost all worms within 24 h. Male worms seemed more susceptible than female under these conditions. Cumulative egg output of surviving worm pairs *in vitro* was considerably reduced when exposed to the extract. For example, after 4 days of exposure to 50 mg l^{-1}, cumulative egg output was only 0.38 eggs per worm pair compared with 36.35 for untreated worms. *In vivo* efficacy of the extract was tested by oral and subcutaneous delivery of 150 mg kg^{-1} followed by assessment of worm survival and fecundity. Neither delivery route produced any significant reduction in worm numbers compared with untreated controls. Worm fecundity was assessed *in vivo* by cumulative egg counts per liver at 55 days post infection with mice treated subcutaneously. Such infections showed egg levels in the liver of about 2000 eggs per worm pair in 55 days, in both treated and control mice, with no significant difference between the two groups. To ensure that density-dependent effects did not confound this analysis, a separate experiment demonstrated no such influence on egg output per worm pair, at intensities between 1 and 23 worms per mouse.

Introduction

The frequency of severe morbidity resulting from infection with schistosomes has been successfully reduced in many endemic areas by the use of chemotherapy (Salvioli *et al.*, 1997; WHO, 1997). Rapid re-infection usually occurs, however, particularly in schoolchildren, even when treatment of the population has been supplemented with methods aimed at reducing transmission, such as the use of molluscicides, health education and the installation of sanitary facilities (Sleigh *et al.*, 1981; Polderman & De Caluwe, 1989; Chandiwana *et al.*, 1991; Etard *et al.*, 1995; De Clercq *et al.*, 1999).

This state of affairs has led to many schistosomiasis research programmes focused on the search for a vaccine. Currently, however, there are no clinically validated vaccines available for any of the helminth diseases affecting man, although several promising vaccine antigens have been identified in experimental models (Butterworth, 1994; Berquist, 1995). In a recent WHO co-ordinated programme, some of these antigens were tested simultaneously, using the same host/parasite strains. The results were disappointing and the aim of achieving a consistent reduction in worm burden of at least 40% compared to non-immunized controls, was not attained with any of the antigens selected (Gryseels, 2000). This partial degree of protection, if replicated in the human host, might result in a reduction in morbidity but would not necessarily have a great impact on transmission. In such circumstances, the costs of diagnosis and chemotherapy could be lower than the cost of immunization (Guyatt & Evans, 1995; Gryseels, 2000)

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In the absence of a vaccine, chemotherapy will continue to play a central role in schistosomiasis control programmes. At present, praziquantel is the only chemotherapeutic compound recommended for the treatment of both urinary and intestinal schistosomiasis (Salvioli *et al.*, 1997; WHO, 1997). Metrifonate and oxamniquine are less widely used due to a lack of availability, poor cure rates in the field and the demonstration of drug-induced resistance in laboratory models (Cioli *et al.*, 1993; Brindley, 1994; Cioli, 1998).

The potential for the development of resistance to praziquantel was highlighted in 1995 by its apparently low efficacy when used to treat a newly established focus of *Schistosoma mansoni* in Senegal (Stelma *et al.*, 1995). More recent evidence has suggested that the treatment failures observed might have been due, at least in part, to particularly intense transmission resulting in large numbers of non-susceptible immature worms present at the time of treatment (Fallon, 1998; Picquet *et al.*, 1998; Kusel & Hagan, 1999). Field isolates obtained from Senegal have been shown, however, to be more tolerant to praziquantel treatment when compared to other strains maintained in laboratory mice (Fallon *et al.*, 1995). In addition, drug-specific resistance to praziquantel has been induced in a laboratory strain of *S. mansoni* by repeated exposure to sub-curative doses (Fallon & Doenhoff, 1994; Pereira *et al.*, 1998). It is therefore clear that in order to ensure the effective drug control of schistosomiasis in the future, the identification of new and effective schistosomicidal compounds is essential.

Higher plants provide a vast source of novel chemical structures that can be screened for chemotherapeutic activity as well as the potential to be used in the development of cheap and reliable phytotherapeutic preparations (Anon., 1994; Cragg *et al.*, 1997; Xiaorui, 1999; Calixto, 2000). The latter option would be of particular relevance in developing countries where, due to lack of funds, up to 80% of the population relies solely on traditional plant remedies for their primary health care needs (Xiaorui, 1999; Calixto, 2000). Many plant species have been used throughout the world in traditional medicine for the treatment of both veterinary and human helminths (Hammond *et al.*, 1997) but few plants have been screened for activity against adult *S. mansoni.*

Rhizomes of ginger (*Zingiber officinale*) are cultivated in India, South-east Asia, China and Africa and extracts of them have been shown to have activity against *S. mansoni* miracidia and cercariae (Adewumni *et al.*, 1990; Sanderson, 1998). The primary aim of the work presented in this paper was to determine the effect of such an extract on the survival of adult *S. mansoni* both *in vitro*, and by oral and subcutaneous treatment of infected mice. A secondary aim was to investigate extract bioactivity towards adult schistosome fecundity, *in vitro* and *in vivo*.

Schistosome egg output *in vivo* is usually determined by counting the numbers of eggs recovered from urine, faeces and/or host tissues. Estimation of faecal egg counts can be highly inaccurate and the egg output of an individual host can vary on a daily basis (Barreto *et al.*, 1990). In addition, some authors have suggested that schistosome fecundity is density-dependent, the egg output of individual worm pairs being reduced in those hosts with higher worm burdens (Cheever *et al.*, 1987, 1994b; Jones *et al.*, 1989;). In order to address these issues, the approach used in this investigation has been to assess worm fecundity, after subcutaneous treatment of mice, by counting the total numbers of eggs present in the livers at day 55 post-infection. To ensure that possible density-dependent effects did not invalidate the use of this measure, an analysis was carried out, in untreated mice, of the relationship between worm density and liver egg counts.

Materials and methods

Extraction of ginger rhizomes

Fresh ginger rhizomes were sliced thinly and dried in an oven at 45°C for 3–5 days. The dried material was finely ground and 100 g was placed in a Whatman cellulose extraction thimble. Extraction was carried out in a Sohxlet apparatus with 500 ml ethyl acetate for 4 h and the resulting extract was dried using rotary vacuum evaporation at 50°C.

In vitro *bioassays*

The life-cycle of *S. mansoni* was maintained essentially as described by Standen (1949). Mice carrying an infection of 8 weeks were killed by cervical dislocation and dipped in 10% ethanol to minimize contamination. Adult worm pairs were removed aseptically from the hepatic portal and mesenteric veins at laparotomy. They were then placed in a sterile Petri dish with 10 ml of Eagles MEM containing 10% FCS, 0.15% sodium bicarbonate, 2 mM L-glutamine, 1 mM sodium pyruvate and 50 U ml^{-1} penicillin-streptomycin (Sigma-Aldrich, UK). All worm pairs were examined under a dissection microscope and healthy undamaged pairs were placed in a sterile test-tube and washed five times with medium. For each of the concentrations of the ginger extract tested, one worm pair was transferred aseptically to each of five wells of a sterile Linbro-12 multiwell plate (ICN - UK) containing 2.97 ml of medium. Thirty μl of the ginger extract dissolved in ethanol was added to give final concentrations of 200, 100, 50, 25 and 12.5 mg l^{-1} and 30 μl of ethanol was used for comparable control worms. The plates were then incubated in a 4% CO_2 atmosphere at 37°C for 4 days. On days 1, 2 and 4 of the incubation, the percentage survival of male and female worms was assessed and the number of eggs that had been laid was counted. In total, seven replicate assays were carried out.

Determination of the relationship between worm burden and egg deposition in the liver

Cercariae were collected by illumination of infected snails under a 60-watt lamp for 2 h and diluted to a density of 20 ml^{-1}. Further serial dilutions of the cercarial suspension were made to give five aliquots having densities of 20, 15, 10, 5 and 2.5 cercariae ml^{-1}. For each aliquot, 10 ml of the cercarial suspension was used to infect each of 10 mice by the paddling method Standen (1949). Thus five groups of 10 mice were infected with 200, 150, 100, 50 and 25 cercariae per mouse. All mice were autopsied on day 55 post-infection and the numbers

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of adult worm pairs were counted as described by Standen (1949) by direct examination of mesenteric blood vessels. Each liver was squashed between two glass plates and examined for live and dead worms under low power (× 50). The number of eggs present in the liver of each mouse was determined using a modification of the method described by Cheever *et al.* (1987). Briefly, each of the livers squashed at autopsy was shaken in 300 ml of 4% potassium hydroxide and incubated at 37°C until digestion of the liver tissue was complete (approximately 18 h). The eggs were then allowed to settle for 2 h after which the top 200 ml was removed using a peristaltic pump. The eggs were then re-suspended and counts were carried out on five 150 μl samples using McMaster slides. The egg output per worm pair was then calculated.

In vivo *experiments*

Twenty female NMRI mice were infected with 150 cercariae using the paddling method. On each of days 49–53 post-infection, 10 mice were dosed either subcutaneously or orally by gavage with 150 mg kg^{-1} of the ginger extract (0.05 ml of a 30 mg l^{-1} solution in 5% ethanol, per 10 g body weight). A group of 10 mice was treated in a similar manner with 5% ethanol and used as controls. All mice were killed by cervical dislocation on day 55 post-infection and the number of adult worms was counted. Liver egg-counts were only carried out on mice that had been dosed by subcutaneous injection.

Statistical analysis

The *P*-values quoted were calculated using the unpaired, two sample Student's t-test. Statistical analysis was carried out on the arcsine values for percentage data to ensure normality of distribution (Alder & Roessler, 1976).

Results

Experiments in vitro

The percentage survival of male and female *S. mansoni* worms following *in vitro* exposure to a range of concentrations of the ginger extract is shown in fig. 1. By 24 h, all male and 99.6% of female worms exposed to 200 mg l^{-1} were killed, whereas no deaths were observed in the controls ($P < 0.001$). Figure 1 suggests that male worms were slightly more susceptible to the extract than the females and this was most obvious for those worms that were exposed to 100 mg l^{-1} of the extract. These observed differences were not, however, statistically significant except for worm pairs that were exposed to 100 mg l^{-1} for 24 h ($P < 0.05$).

By day 4 of the bioassay, 73% of the control worm pairs had produced eggs, with individual counts ranging from 1 to 197. The cumulative numbers of eggs per worm pair, calculated only for those cultures containing live male and female worms, are shown in fig. 2. At sub-lethal concentrations of the ginger extract, the egg output of surviving worms was significantly reduced. For example, after a 4-day exposure to 50 mg l^{-1}, approximately 60% of the worm pairs that were tested survived. These worms however, gave a mean cumulative egg count after 4 days of only 0.38 eggs per worm pair compared to 36.35 for control worms ($P < 0.01$).

Experiments in vivo

No significant difference was observed in the mean worm burden of host mice, following five consecutive daily treatments of 150 mg kg^{-1} of the ginger extract, administered either orally ($t = -1.29$, $P = 0.213$, $DF = 18$) or subcutaneously ($t = 0.98$, $P = 0.338$ $DF = 20$), compared to infected control mice, as shown in fig. 3.

In order to assess the *in vivo* effects of the ginger extract on the fecundity as well as survival of adult *S. mansoni*,

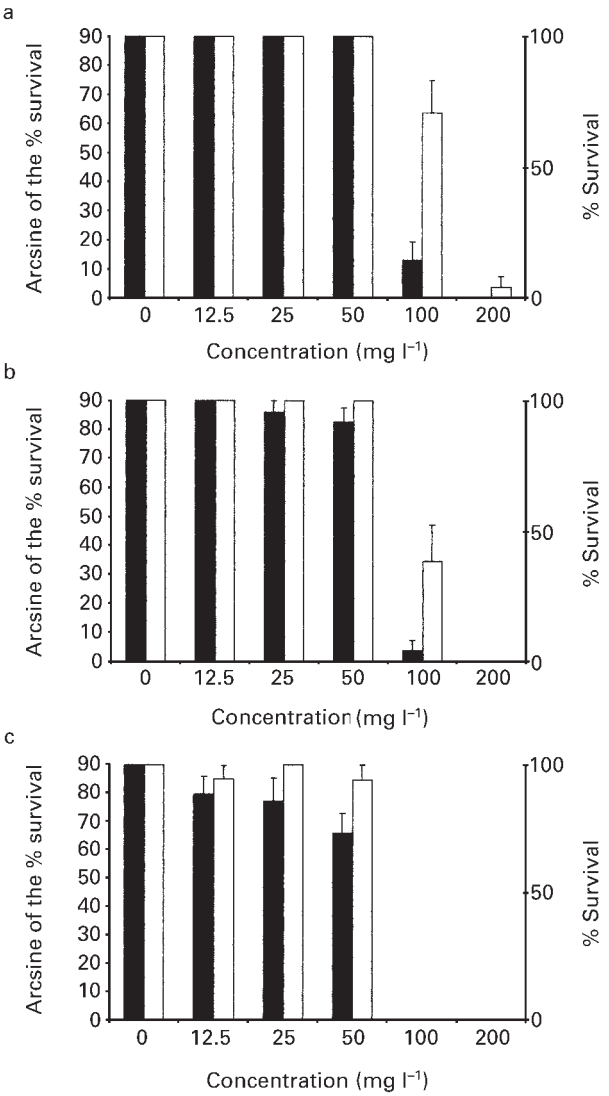

Fig. 1. The effect of an ethyl acetate extract of ginger on the survival of adult male (■) and female (□) *Schistosoma mansoni* in vitro after (a) 24 h, (b) 48 h and (c) 4 days. Histogram bar heights for (a) and (b) represent the mean of seven replicate assays and for (c) represent the mean of five replicate assays. Error bars represent the standard error.

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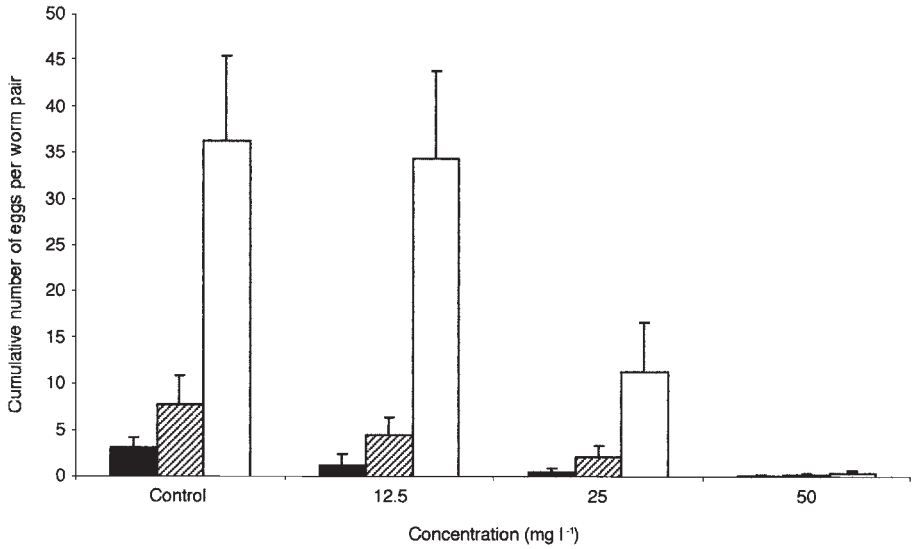

Fig. 2. The effect of an ethyl acetate extract of ginger on the cumulative egg output of live worm pairs of *Schistosoma mansoni* cultured *in vitro* for 4 days. Histogram bar heights represent the mean of not more than 36 and not less than 16 worm pairs. Error bars represent the standard error. ■, 24 h; ▨, 48 h; □, 4 days.

the relationship between adult worm burdens and egg deposition in the liver was first studied. Figure 4 shows a positive and linear correlation between worm burden and the total number of eggs in the liver on day 55 post-infection. The correlation coefficient was calculated to be 0.945 and the slope of the line, representing the theoretical mean number of eggs per worm pair deposited in the liver by day 55, was calculated to be 2156. On the basis of these results, it was concluded that this variable could be used as an indicator of schistosome fecundity for the host-parasite combination used in this study. In addition, a direct comparison could be made between treated and untreated host mice irrespective of any difference in their infection intensities.

The effect of the ginger extract on the number of eggs in the liver per worm pair at day 55 post-infection is shown in fig. 5. No significant difference was observed between control mice and those treated subcutaneously with five consecutive daily treatments of 150 mg kg^{-1} ($t = 1.00$, $P = 0.328$, $DF = 20$). The mean number of liver eggs per worm pair in untreated mice in this experiment (2116) was strikingly similar to that calculated from the data for untreated mice with widely varying worm loads (2165) ($t = 1.01$, $P = 0.317$, $DF = 46$). Quantitative estimates of liver eggs were not carried out on mice that were dosed by the oral route but the macroscopic appearance of the livers did not suggest a reduction in egg output.

Discussion

The *in vitro* bioassay clearly showed that ginger rhizomes contain one or more compounds that are active against adult *S. mansoni* and it would appear that these compounds act preferentially against male rather than female worms. A similar variation in drug susceptibility between male and female schistosomes has been observed with several antischistosomal drugs (Popiel & Erasmus, 1982; Fallon *et al.*, 1994).

As well as sustaining adult worms for at least 4 days, the *in vitro* culture conditions employed in this study permitted oviposition (with 73% of the untreated females having produced eggs by day 4) allowing direct observation of the effects of the ginger extract on egg production. Although the cumulative egg output of individual worm pairs was highly variable, wide variations in the egg output of individual worm pairs have been demonstrated by other authors, both *in vitro* (Schirazian & Schiller, 1983; El-Ridi *et al.*, 1997) and *in vivo* (Cheever *et al.*, 1994a). Despite this variability, the *in vitro* bioassay showed a significant reduction in the mean egg output of surviving females following exposure to sub-lethal concentrations of the ginger extract. It is not known whether these anti-fecundity effects were the result of generalized cytotoxic damage or more specific inhibition of reproductive processes by one or more of the compounds present.

In order to estimate the effects of the extract on worm fecundity *in vivo*, a comparison of the mean numbers of eggs present in the liver per worm pair, of the treated and untreated host mice was carried out. For some strains of mouse, it has been reported that the numbers of eggs per worm pair, in both the tissues and faeces, decrease with increasing infection intensity (Cheever *et al.*, 1987, 1994b; Jones *et al.*, 1989), giving rise to the hypothesis that schistosome fecundity *in vivo* may be density-dependent. The results of the experiment to define this relationship showed no such density-dependent inhibition for the host/parasite system used in this study. On the basis of these results, it was concluded that the mean number of eggs present in the liver per worm pair, on day 55 post-infection, could be used as an indicator of schistosome

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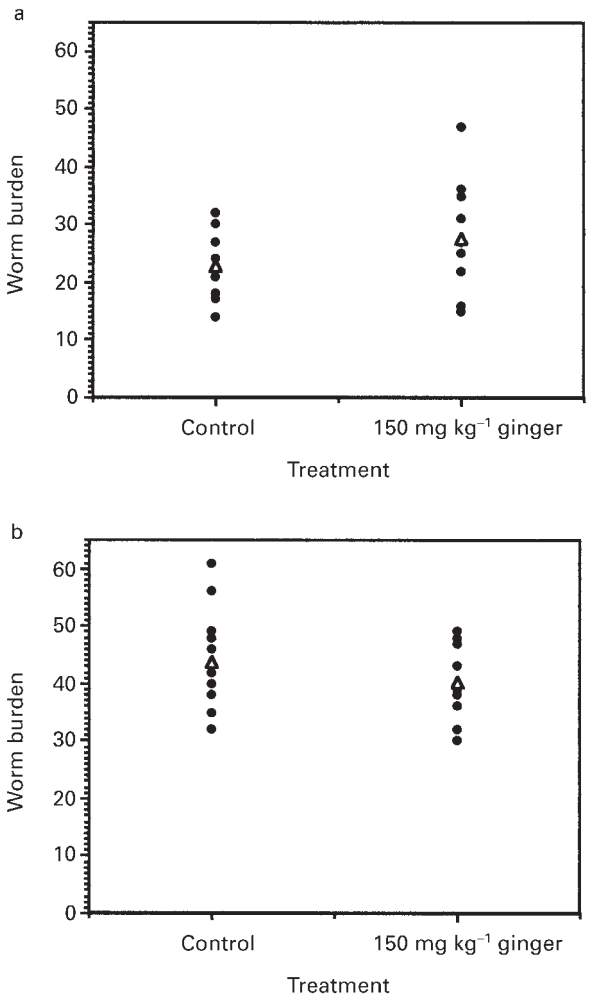

Fig. 3. The effect of five consecutive daily treatments via (a) oral and (b) subcutaneous administration with an ethyl acetate extract of ginger, on adult *Schistosoma mansoni* worm burdens in mice (49–53 days post-infection). ● Data points represent the worm burdens for individual mice. △ Data points represent the mean worm burden of 10 mice.

fecundity and that a direct comparison of this variable could be made between treated and untreated host mice, irrespective of any difference in their infection intensities.

The antischistosomal activity of the ginger extract that was observed *in vitro* could not be reproduced *in vivo* using the dosage schedules described. The active compound(s) present in the ginger extract therefore failed to reach the mesenteric blood vessels in concentrations that would be sufficient to kill adult schistosomes. This could be due to one or a combination of factors, probably the most significant of which was the administration of an inadequate dose. The total dosage of the ginger extract that could be administered over 5 consecutive days to mice carrying a patent infection was limited to 750 mg kg^{-1} as it had to be delivered in 5% ethanol. The individual compound(s) responsible for the activity that was observed *in vitro*, were therefore tested at lower dosages than those commonly used for the currently

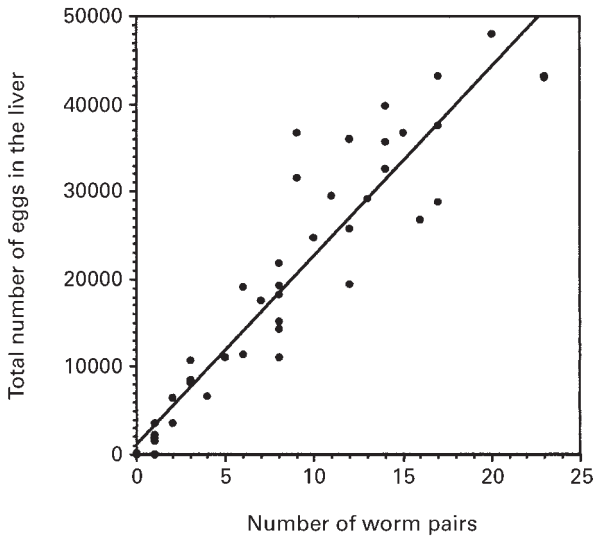

Fig. 4. The relationship between the number of adult *Schistosoma mansoni* worm pairs present in the hepatic portal system and the total number of eggs in the liver on day 55 post-infection. Data points represent the number of adult worm pairs vs. total number of eggs in the liver for individual mice. $y = 1148.8 + 2156.2 \times (r = 0.945)$.

available schistosomicides. For example, total dosages of 750–1000 mg kg^{-1} of praziquantel have been reported to be required to kill patent *S. mansoni* infections in host mice (Melhorn *et al.*, 1981; Sabah *et al.*, 1986). The limitations imposed on the dosage of active compound(s)

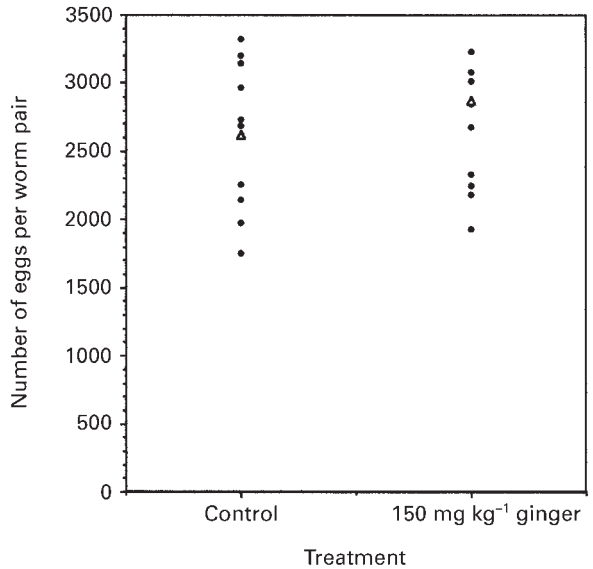

Fig. 5. The effect of five consecutive daily treatments with an ethyl acetate extract of ginger on the fecundity of adult *Schistosoma mansoni* in mice, as measured by the number of eggs in the liver. Ginger was administered sub-cutaneously on days 49–53 post-infection. ● Data points represent the number of eggs per worm pair for individual mice. △ Data points represent the mean number of eggs per worm pair for 10 mice.

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which can be administered via a crude phytotherapeutic preparation, only serve to exacerbate the difficulties that are usually faced when attempting to deliver a potential drug to its site of action. These include degradation by gastric acid or digestive enzymes in the alimentary tract, poor absorption across the intestinal wall, adsorption by serum proteins or first pass metabolism in the liver and other organs. At present it is not clear to what extent, if any, these factors contributed to the failure to achieve sufficient serum concentrations of active compound(s) in the mesenteric and portal vessels.

Whilst these results suggest that crude preparations of the ginger extract would not be suitable for the treatment of schistosomiasis, isolation of the compounds responsible for the activity observed *in vitro* could result in identification of compounds with potential chemotherapeutic efficacy. It would then be necessary to test the compounds when delivered by oral or parenteral routes. Even if such testing failed to show useful activity, the fact that the original extract, containing those molecules, kills worms *in vitro* would justify further attempts to ensure that they reached the microhabitat of worms *in vivo* at therapeutic concentrations. This could be done either by adapting the delivery formulation or by modifying the molecules themselves to enable them to surmount permeability barriers.

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