In vitro antifilarial activity of extracts of the medicinal plant *Cardiospermum halicacabum* against *Brugia pahangi*

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Abstract

The *in vitro* effects of ethanol and aqueous extracts of the medicinal plant *Cardiospermum halicacabum* on adult worms and microfilariae of *Brugia pahangi* were investigated. With or without the plant extracts in culture medium, the motility of adult worms, microfilariae and microfilarial release from female worms were monitored daily. After 7 days of culture, viability or tissue damage of adult worms was assessed using the MTT assay. At $>500\,\mu g\,ml^{-1}$, the aqueous extract significantly reduced motility of adult females after 24 h of exposure and adult males after 3 days. The aqueous extract, at $>500\,\mu g\,ml^{-1}$, also significantly reduced microfilarial release from female worms, starting on day 2. The reduction in the motility of adult worms and the pattern of microfilarial release from female worms were concentration and time dependent. The MTT assay results revealed that adult worms cultured in the presence of aqueous extracts at $>500\,\mu g\,ml^{-1}$ were damaged. However, the aqueous extract did not affect the motility of microfilariae with the exception of those in higher concentration extracts. Higher concentrations of ethanol extracts ($2\,mg\,ml^{-1}$) inhibited both the motility of adult worms and the release of microfilariae from females. Little effect of ethanol extracts was detected by the MTT assay, as only slight damage was caused to worms exposed only to the highest concentration ($2\,mg\,ml^{-1}$). However, ethanol extract at $500\,\mu g\,ml^{-1}$ rapidly reduced the motility of microfilariae on day 2. The present study revealed that an aqueous extract of *C. halicacabum* has mild but definite direct macrofilaricidal action on *B. pahangi*.

Introduction

Filariasis causes major public health and socio-economic problems in tropical areas. Recently, WHO (1995) estimated that 120 million people in at least 73 countries suffer from lymphatic filariasis; and 1.1 billion people living in endemic areas are at risk of infection. Two drugs, diethylcarbamazine and ivermectin, are available for the control of filariasis and both are known to be effective against microfilariae. However, only diethylcarbamazine is considered effective for killing adult worms in lymphatic filariasis (WHO, 1995). Because diethylcarbamazine causes severe side effects, the development of safer and more effective macrofilaricides is needed. Numerous drugs have been discovered through research on medicinal plants used by local healers. Therefore, efforts have been made to screen medicinal plants for antifilarial activities. Comley (1990) reviewed a list of plants which have been investigated for potential use in the treatment of filariasis. A few promising drugs have been developed. *Cardiospermum halicacabum* (Sapindaceae) is an annual herbaceous creeper, which flowers all year round. The entire plant is used internally and externally for the treatment of swelling and inflammation (Sadique *et al*., 1987; Khunkitti *et al*., 1998). It has

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been used for centuries by local healers in Thailand for treatment of inflammation as well as skin worm disease (probably gnathosthomiasis). In Sri Lanka, this plant is traditionally used for its anthelmintic properties (Jayaweera, 1982). However, it has not yet been investigated for its anthelmintic activity. Therefore, the present study was undertaken to examine the efficacy of ethanol and aqueous extracts of *C. halicacabum* against filarial worms of *Brugia pahangi* in vitro.

Materials and methods

Chemicals

NCTC-135, Iscove's Modified Dulbecco's medium (IMDM) and 3-(4,5 diethylthiazole-2-ly)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemical Co., USA. RPMI 1640 was from Gibco, USA. Other chemicals were obtained from Wako Pure Chemicals, Japan.

Preparation of C. halicacabum *plant extracts*

Specimens of *C. halicacabum* were collected from Khon Kaen province, Thailand. Aerial parts of the plant were cleaned and dried at 50°C for 8 h. Subsequently, the dried plants were cut into small pieces and ground to a powder, which was stored in a desiccator at room temperature until use.

Aqueous extraction

The 500 g of ground plant were boiled in 2 l purified water until the aqueous volume was reduced to approximately 1 l. After the aqueous extract was cooled, the filtrate, collected by muslin filtration, was again filtered by vacuum filtration prior to freeze-drying.

Ethanol extraction

The 500 g of ground plant was macerated in 2 l of 95% ethanol. After 7 days of maceration, the ethanol extract was collected by filtration in the same manner as described for the aqueous extract. The ethanol was removed by a rotational evaporator. The residue was collected and freeze-dried.

Preparation of culture medium, test solutions and parasites

The culture medium used in this study was a 1:1 mixture of NCTC-135 and Iscove's Modified Dulbecco's medium (IMDM) containing 0.01% w/v Tween 80 (NIT). A stock solution of the ethanol or aqueous extracts was prepared in NIT medium and adjusted to pH 7.4 to give a final concentration of 2 mg ml^{-1}. The solution was then sterilized by membrane filtration. The stock solution was subsequently diluted with NIT medium to give the required final concentrations of 1000, 500, 100 and 10 μg ml^{-1}, respectively. *Brugia pahangi* infected jirds used in this study were obtained from the Animal Center of the Institute of Tropical Medicine, Nagasaki University, Japan. Adult filarial worms were recovered from the peritoneal cavity of jirds 9 months after being infected peritoneally with 200–300 infective larvae. They were washed three times in sterile NIT medium (pH 7.4) before male and female worms were separated. All males were transferred as a mass to 6 ml of NIT medium supplemented with 10% heat inactivated foetal bovine serum (hiFBS) and cultured for 24 h at 37°C in 5% CO_2 in air prior to exposure to the extract. Females were transferred singly into each well of 24-well plates (Sumilon, Sumimoto Bakelite, Japan) containing 1 ml of NIT medium supplemented with 10% hiFBS. The plates were then incubated for 24 h at 37°C in 5% CO_2 in air. Only those females which were active (i.e. moving freely) and released more than 1000 microfilariae over 24 h of incubation were selected for subsequent study. Microfilariae were collected from the peritoneal cavity of the infected jirds and purified through a PD-10 column as described by Galal *et al*. (1989). The purified microfilariae were washed three times with sterilized NIT medium containing 500 units ml^{-1} streptomycin and 500 μg ml^{-1} penicillin. After washing and centrifugation at 700 *g* for 2 min, the pellet was re-suspended and adjusted to a final density of one million microfilariae ml^{-1}.

Efficacy of plant extracts on adult worms

The selected males and females were transferred singly into each well in 24-multiwell plates containing 0.1 ml of hiFBS and 0.9 ml of culture medium with or without the extract. Each experiment was performed in six replicates. The motility, viability, and microfilarial release from adult worms were examined. The motility of males and females were observed daily for 7 days under an inverted microscope. The worm's motility was recorded using an arbitrary score of 3 (highly active), 2 (moderately active), 1 (less active) and 0 (immobile for at least 10 sec). The results were expressed as the average motility score with respect to the exposure time. Immediately after a daily motility score was recorded, female worms were transferred into sample media containing fresh extract and the number of microfilariae released into the culture media over a period of 24 h was counted. The number of microfilariae in two 10 μl aliquots of culture media was determined, and the total number of microfilariae released into 1 ml medium calculated by multiplying the average of these values by 100. Results were expressed as the percentage pre-treatment level of microfilariae released. At the end of the experiment (7 days), the viability of each worm was examined using the MTT assay (Comley *et al*., 1989a) and the results expressed as the average absorbance values per unit worm length (cm).

Efficacy of plant extracts on microfilariae

A 10 μl aliquot of the microfilariae suspension was transferred into each well of 24-well plates which contained 0.1 ml of hiFBS and 0.9 ml of the plant extracts in NIT medium at various concentrations (0, 100, 500, 1000 and 2000 μg ml^{-1}). Each experiment was performed in six replicates. The plates were incubated at 37°C in 5% CO_2 in air for 7 days. Both motile and immotile worms in 10 μl aliquots of each sample were counted daily without changing the medium for 7 days. Microfilariae, which did not move for 10 sec, were considered as immotile, i.e. non-surviving. Results were expressed as the percentage of motile parasites in extract-treated incubated to the

percentage of mobile parasites in the same wells prior to treatment.

Statistical analysis

Results were expressed as mean ± SD. Scheffes' test was used to compare the differences between treated and untreated groups for the purpose of adjusting multiple comparisons (Amitage & Berry, 1994). Each experiment was conducted in six replicates. The statistical analysis was interpreted at a *P*-value of 0.05.

Results

Motility of adult worms

Untreated adult females and males remained highly active in NIT medium throughout the experiment (figs 1 and 2). The motility of female worms treated with the aqueous extract at concentrations greater than 500 $\mu g\,ml^{-1}$ gradually reduced as the exposure time increased, whereas lower concentrations did not affect motility (fig. 1a). The motility of male worms appeared to decrease in a concentration- and time-dependent fashion. None of the adult worms were completely immobilized by day 7 (fig. 1b). The effect of ethanol extract on worm motility is shown in fig. 2. The motility of male worms gradually decreased by day 5 and remained sluggish by day 7, whereas the females treated with 2 $mg\,ml^{-1}$ of the extract were less active by day 2, and completely immobilized by day 7. At lower concentrations of the ethanol extract, detectable effects on male or female worm motility were evident.

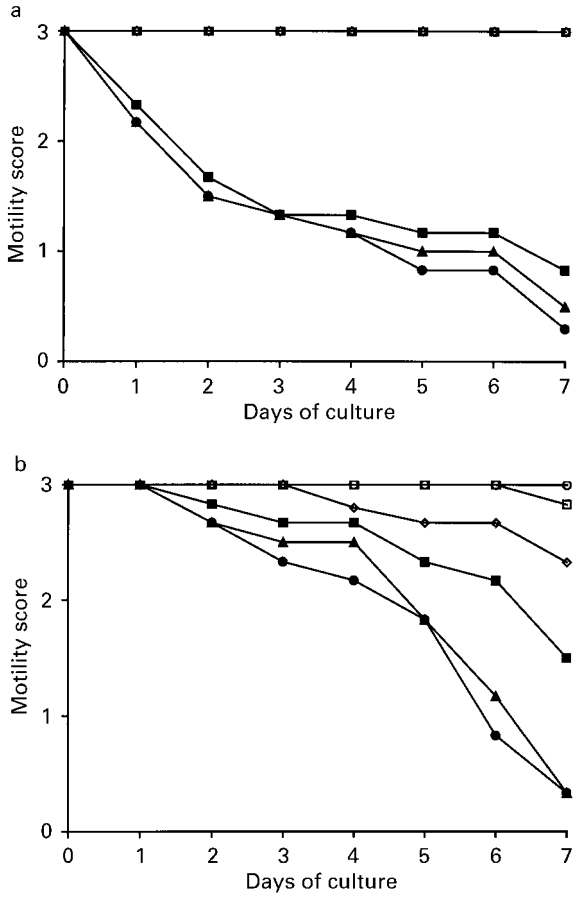

Fig. 1. Motility of females (a) and males (b) of *Brugia pahangi* exposed to aqueous extracts of *Cardiospermum halicacabum*. ○, Control; ●, 2000 $\mu g\,ml^{-1}$; ▲, 1000 $\mu g\,ml^{-1}$; ■, 500 $\mu g\,ml^{-1}$; ♦, 100 $\mu g\,ml^{-1}$; □, 10 $\mu g\,ml^{-1}$.

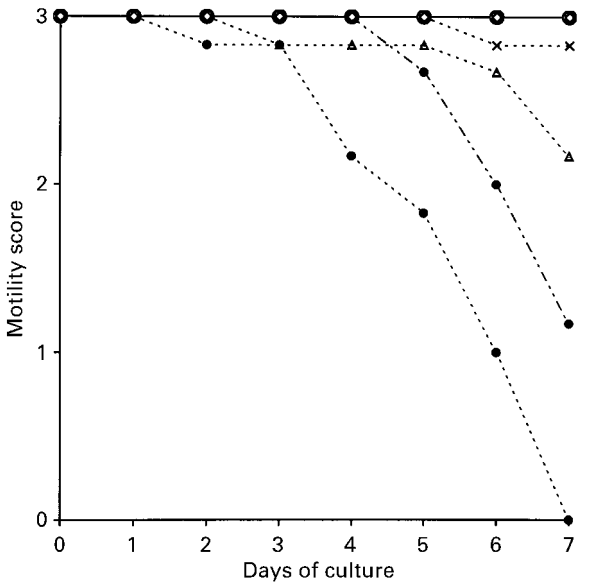

Fig. 2. Motility of females and males of *Brugia pahangi* exposed to ethanol extracts of *Cardiospermum halicacabum*. ○, Control; ●, 2000 $\mu g\,ml^{-1}$; △, 1000 $\mu g\,ml^{-1}$; ×, 500 $\mu g\,ml^{-1}$; □, 100 $\mu g\,ml^{-1}$; ◇, 10 $\mu g\,ml^{-1}$. Solid, dashed and dash-dotted lines represent motility scores of control, treated males and treated-females, respectively.

Microfilarial release by females

Untreated females showed a relatively stable release of microfilariae for up to 5 days and this release markedly decreased thereafter (fig. 3). The aqueous extract, at concentrations greater than 500 $\mu g\,ml^{-1}$ significantly inhibited microfilarial release within 4 days of incubation (fig. 3a). At 100 $\mu g\,ml^{-1}$, the number of microfilariae released gradually declined; whereas at 10 $\mu g\,ml^{-1}$, there was no significant difference in microfilarial release relative to the controls (fig. 3a). At a concentration of 2 $mg\,ml^{-1}$ of the ethanol extract, microfilarial release was significantly less than controls by day 3, and completely interrupted by day 5 (fig. 3b). Lower concentrations of the ethanol extract showed no significant effect on microfilarial release.

MTT assay

Results of MTT assays on adult worms cultured for 7 days are shown in fig. 4. The average length of female and male worms in this study was 4.1 ± 0.3 cm and 1.9 ± 0.1 cm, respectively. Female worms treated with the aqueous extract at the concentrations of 2, 1 and 0.5 $mg\,ml^{-1}$ and the ethanol extract at 2 $mg\,ml^{-1}$ showed

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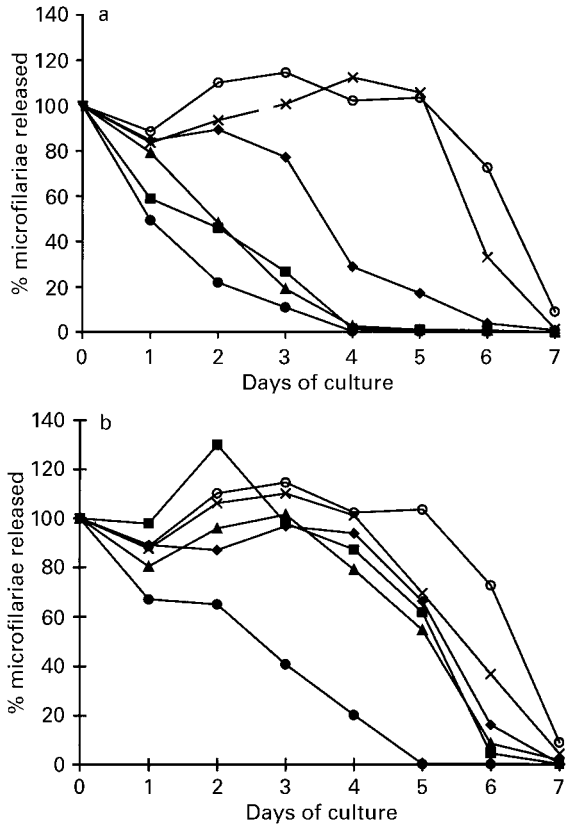

Fig. 3. Microfilarial release by females of *Brugia pahangi* cultured with aqueous (a) and ethanol (b) extracts of *Cardiospermum halicacabum*. ○, Control; ●, 2000 μg ml^{-1}; ▲, 1000 μg ml^{-1}; ■, 500 μg ml^{-1}; ♦, 100 μg ml^{-1}; ×, 10 μg ml^{-1}.

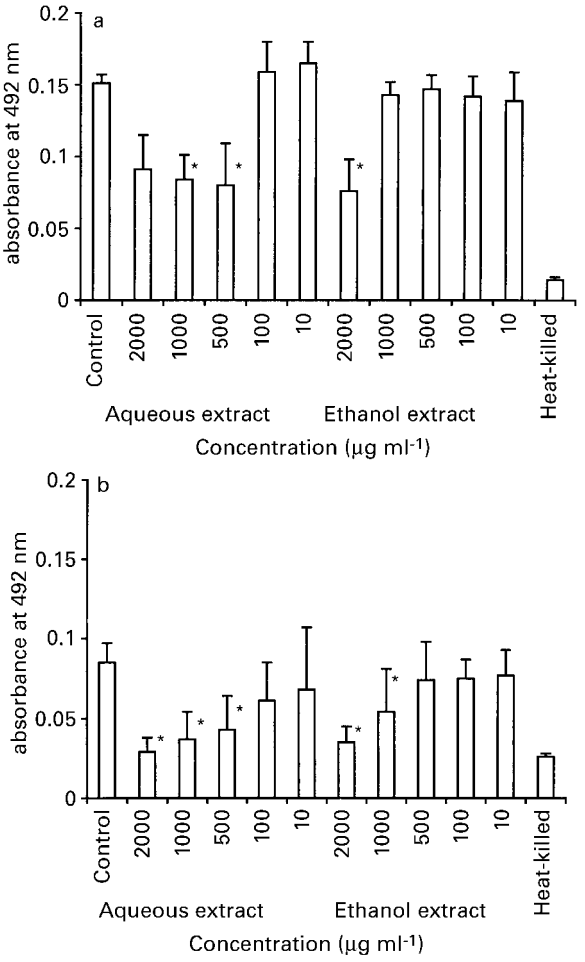

Fig. 4. Absorbance of formazan in DMSO per cm at 492 nm of *Brugia pahangi* females (a) and males (b) after cultured with ethanol and aqueous extracts of *Cardiospermum halicacabum* for 7 days. *Significantly different from control and heat-killed worms (Scheffe's test, $P < 0.05$).

significantly less absorbance values than that of untreated female worms (fig. 4a). However the values were significantly higher than that of heat killed female worms. A low absorbance was recorded in males treated with 2, 1 and 0.5 mg ml^{-1} of the aqueous extract and 2 and 1 mg ml^{-1} of the ethanol extract (fig. 4b).

Microfilarial motility

Control groups were actively motile for only 3 days and motility decreased thereafter (fig. 5). For the aqueous extract, the highest concentration (2 mg ml^{-1}) showed that approximately 50% of treated microfilariae were immobile on day 3 and completely immobile on day 5 (fig. 5a). Concentrations lower than 2 mg ml^{-1} were less effective. The ethanol extract at 2 and 1 mg ml^{-1} inhibited the motility of microfilariae within 2 days of incubation, whereas a concentration of 0.5 mg ml^{-1} required at least 4 days to significantly reduce the motility of the microfilariae. Concentrations lower than 0.5 mg ml^{-1} were comparable to untreated controls (fig. 5b).

Discussion

The present study was designed to examine the *in vitro* efficacy of *C. halicacabum* extracts on the filarial worm, *B. pahangi*. The culture method used allowed adult worms to be actively motile for 7 days, and to release microfilariae for 5 days; the microfilariae were motile for 3 days. Although the osmolarity of the culture medium with high concentrations of the plant extract was slightly higher (314 mOsmol l^{-1}) than that of culture medium without extract (301 mOsmol l^{-1}), our preliminary experiments revealed that a slight change in osmolarity does not affect the motility of adult worms nor the microfilariae.

In our study, both aqueous and ethanol extracts of *C. halicacabum* were tested. Efficacy of the extracts on *B. pahangi* was evaluated by motility and a MTT assay using male and female worms, microfilarial release from female worms, and the motility of microfilariae. The motility of adult worms cultured in the presence and absence of plant extracts was compared. Both aqueous and ethanol extracts of *C. halicacabum* inhibited the motility of microfilariae and adult worms at relatively high concentrations. This finding was similar to that already shown for several other medicinal plants with

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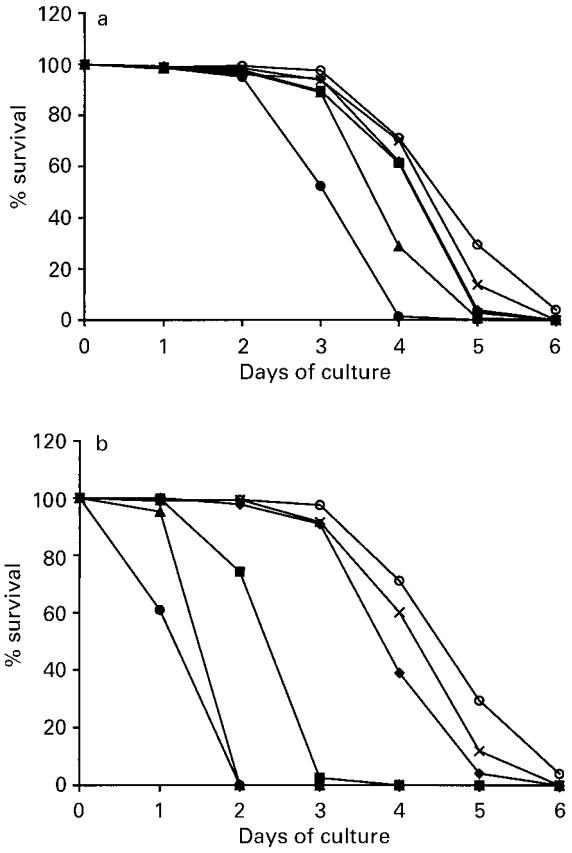

Fig. 5. Survival of microfilariae of *Brugia pahangi* exposed to aqueous (a) and ethanol (b) extracts of *Cardiospermum halicacabum*. ○, Control; ●, 2000 $\mu g\,ml^{-1}$; ▲, 1000 $\mu g\,ml^{-1}$; ■, 500 $\mu g\,ml^{-1}$; ♦, 100 $\mu g\,ml^{-1}$; ×, 10 $\mu g\,ml^{-1}$.

antifilarial activities (Comley *et al*., 1990). This suggests that filaricidal compounds may be present at low concentrations in crude extracts of these plants, or that the compounds are of low intrinsic potency, or both. Although a relatively high concentration of plant extract was required, the plant extract exhibited direct actions on both adult worms and microfilariae. The effect of the extract was concentration- and time-dependent. At concentrations higher than 0.5 $mg\,ml^{-1}$, aqueous extract significantly reduced motility of adult worms and release of microfilariae from female worms. The MTT assay for worm viability is based on the ability of various NADH-dependent dehydrogenase enzymes in viable worms to reduce MTT, a pale yellow, to formazan, a dark blue crystalline. Inhibition of MTT reduction on drug-treated worms may indicate drug-induced damage and may provide an estimate of the degree of impairment (Comley *et al*., 1989a,b; Akao *et al*., 1995). In the present study, the MTT assay revealed that adult worms cultured for 7 days in the presence of aqueous extract at $\geqslant$ 0.5 $mg\,ml^{-1}$ were damaged. The aqueous extract, however, was less effective on microfilariae. Conversely, the ethanol extract was effective against microfilariae, but not adult worms. This pattern of stage-dependent activity was also found in extracts of *Streblus asper* and *Senecio nudicaulis* against *Setaria cervi* in vitro (Parveen *et al*., 1989; Singh *et al*., 1996). This finding suggests that there are differences in the active compounds extracted by two methods, or in the activities of these compounds against adult worms and microfilariae, or their absorption by adult worms and microfilariae.

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