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# Macrofilaricidal and microfilaricidal effects of *Neurolaena lobata*, a Guatemalan medicinal plant, on *Brugia pahangi*

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**Abstract**

Twelve extracts of 11 Guatemalan medicinal plants were initially screened *in vitro* for potential macrofilaricidal activity against *Brugia pahangi*, a lymphatic dwelling filarial worm, using concentrations from 125 to 1000 $\mu g\,ml^{-1}$ of each extract that could be dissolved in the culture medium. Of 12 extracts used, the ethanol extract of leaves of *Neurolaena lobata* showed the strongest activity against the motility of adult worms. Subsequently, the extract of *N. lobata* was extensively examined *in vitro* for macro- and micro-filaricidal effects using a series of concentrations of 500, 250, 100, 50 and 10 $\mu g\,ml^{-1}$. The effects were assessed by worm motility, microfilarial release by female worms and a MTT assay. The effect on the motility of adult worms was observed in a concentration- and time-dependent manner. The time required to stop motility of both sexes of adult worms was 6 h at 500 $\mu g\,ml^{-1}$, 24 h at 250 $\mu g\,ml^{-1}$, and 3 days for females and 4 days for males at 100 $\mu g\,ml^{-1}$. The movement of females ceased at 4 days at a concentration of 50 $\mu g\,ml^{-1}$ whereas the motility of males was only reduced. The loss of worm's viability was confirmed by the MTT assay and was similar to the motility results. These concentrations, including 10 $\mu g\,ml^{-1}$, prevented microfilarial release by females in a concentration- and time-dependent manner. Concentrations higher than 100 $\mu g\,ml^{-1}$ even induced mortality of the microfilariae. The present study suggested that the ethanol extract of *Neurolaena lobata* has potential macro- and micro-filaricidal activities.

## Introduction

Lymphatic filariasis caused by *Wuchereria bancrofti*, *Brugia malayi* and *B. timori* affects 120 million people in over 80 countries (WHO, 2001). Drugs used to cope with the disease are diethylcarbamazine (DEC) and ivermectin. Although both of these have a marked microfilaricidal activity, neither has appreciable action against adult worms. Thus the discovery and development of a safe macrofilaricidal drug for the mass therapy of lymphatic filariasis is still a major objective of Tropical Disease Research (TDR, 1997). Potential sources of new macrofilaricidal drugs are: (i) the synthetic programme within the pharmaceutical industry; and (ii) the molecular biological approach involving the rational design of compounds to the activity site, for example, of a target receptor protein. Other valuable sources are chemicals

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derived from natural products. In the case of filarial infections, higher plants have not been extensively utilized and this resource has been largely uninvestigated. Comley (1990) reviewed a list of 90 plants that have been investigated for potential use in the treatment of filariasis. Several papers have reported on the antifilarial activity of medicinal plants in India (Chatterjee *et al.*, 1992; Parveen *et al.*, 1992; Singh *et al.*, 1996, 1997, 2000; Ghosh *et al.*, 1998), in Thailand (Khunkitti *et al.*, 2000) and in Malaysia (Zaridah *et al.*, 2001). Some of them are included in a list of 16 medicinal plants with activity against filarial worms (Tagboto & Townson, 2001). Few promising drugs, however, have been developed yet.

In the present study, 12 Guatemalan medicinal plant extracts were screened initially for antifilarial activity *in vitro*. Then ethanol extracts of the leaves of *Neurolaena lobata* were investigated in more detail to determine any macro- and micro-filaricidal effects against adult worms of the lymphatic filarial nematode *Brugia pahangi*. This is the first report on antifilarial activity of Guatemalan medicinal plants.

## Materials and methods

### *Chemicals and plant extracts*

The NCTC135 medium, Iscove's modified Dulbecco's medium (IMDM), tetracycline hydrochloride (TC) and 3-(4,5-dimethylthiazol-2-ly)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemical Co., USA.

The characterization of 12 Guatemalan medicinal plants extracts, prepared by the method previously described (Cáceres *et al.*, 1998) and used in the present study, is summarized in table 1. Concentrations of each extract tested in the preliminary experiments ranged from 125 $\mu g\,ml^{-1}$ to 1000 $\mu g\,ml^{-1}$, the last being the highest concentration that could be dissolved in the culture medium. Concentrations of the extract of *N. lobata* tested in subsequent experiments were 10, 50, 100, 250 and 500 $\mu g\,ml^{-1}$.

### *Preparation of the culture medium*

The culture medium used in this study was a 1:1 mixture of NCTC135 and IMDM containing 0.01% Tween 80 (NIT). All the culture media with each plant extract were prepared in NIT and adjusted to pH 7.4. The solution was then sterilized by membrane filtration (Minisart®, pore size 0.20 $\mu$m, Sartorius AG, Goettingen, Germany).

### *Filarial worms*

*Brugia pahangi* used in this study has been routinely maintained in Mongolian gerbils and *Aedes aegypti* at the Animal Center of the Institute of Tropical Medicine, Nagasaki University. The collection and selection of adult worms were carried out following the method of Khunkitti *et al.* (2000). Briefly, adult worms were recovered from the peritoneal cavity of jirds 6–9 months after peritoneal infection with 200 infective larvae and washed with NIT medium. Females were separated from males and transferred singly into wells of a 24-well plate (Sumilon, Sumitomo Bakelite, Japan), containing 1 ml of NIT medium supplemented with 10% heat inactivated foetal bovine serum (hiFBS). Female *B. pahangi* were incubated at 37 °C in 5% $CO_2$ in air. After 24 h incubation, only active females releasing more than 1000 microfilariae (mf) into the medium were selected for subsequent studies. All males were transferred as a mass into 5 ml of NIT medium supplemented with 10% hiFBS and cultured for 24 h. Only active males were selected for subsequent studies.

Microfilariae were collected from the peritoneal cavity of infected jirds and purified through a PD-10 column (Galal *et al.*, 1989). The purified mf were washed three times with sterile NIT medium containing 500 units $ml^{-1}$ streptomycin and 500 $\mu g\,ml^{-1}$ 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 mf per ml.

### *Motility, microfilarial release and MTT assay*

Four females and four males were each allocated to one type of plant extract. Each worm was transferred singly to each well in 24-multiwell plates containing 0.1 ml of hiFBS and 0.9 ml of culture medium with or without extracts. The motility, viability and microfilarial release by females were examined. As previously described by

Table 1. Characterization of Guatemalan medicinal plants tested in the initial experiments.

| Medicinal plants used | Parts of plant extracted | Extracts with | Concentrations tested ($\mu g\,ml^{-1}$) |
|---|---|---|---|
| *Tridax procumbens* | Leaves | Ethanol | 1000 |
| *Acalypha guatemalensis* | Leaves | Ethanol | 1000 |
| *Croton guatemalensis* (1) | Bark | Ethanol | 250 |
| *Croton guatemalensis* (2) | Leaves | Methylene chloride | 125 |
| *Chrysophyllum cainito* | Leaves | Ethanol | 250 |
| *Petiveria alliacea* | ? | Methylene chloride | 125 |
| *Smilax regellii* | Leaves | Ethanol | 167 |
| *Tecoma stans* | ? | Ethanol | 500 |
| *Psidium guajava* | ? | Ethanol | 250 |
| *Gliricidia sepium* | Leaves | Ethanol | 1000 |
| *Rauvolfia tetraphylla* | Root | Ethanol | 1000 |
| *Neurolaena lobata* | Leaves | Ethanol | 500 |

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Khunkitti *et al.* (2000), the motility of females and males was checked daily using arbitrary scores of 3 (highly active), 2 (moderately active), 1 (less active) and 0 (inactive for at least 10 s). In subsequent experiments the motility of both sexes of adult worms was additionally checked at 3, 6, 9, and 12 h and results expressed as the average motility. Immediately after a daily motility score was recorded, each worm was transferred daily to a new well containing fresh culture medium containing extracts. Using the same females, the total number of mf released into 1 ml of medium was calculated and the average for each extract was taken daily for 7 days. Results were expressed as a percentage of the pre-treatment number of mf released. The viability of each worm was assessed by MTT assay (Comley *et al.*, 1989) after 7 days and the results were expressed as absorbance per cm of worm length.

### *Survival of microfilariae*

A 10 $\mu$l aliquot of the mf suspension (approximately ten thousand mf) was transferred into each well of 24-multiwell plates containing 0.1 ml of hiFBS and 0.9 ml of each concentration of the extract. Each of four wells was allocated to each concentration. Plates were incubated at 37°C in 5% $CO_2$ in air. Both motile and immotile worms in 10 $\mu$l aliquot of each sample were counted daily without changing the medium for 7 days. Microfilariae which did not move for 10 s were considered as immotile. Results were expressed as the percentage of motile mf to the total number of both motile and immotile mf.

### *Statistical analysis*

For MTT assay, analysis of variance (ANOVA) was used to compare the differences in absorbance between treated and control groups. A $P$ value of $<0.05$ was considered significant. Each experiment was conducted in three replicates.

## Results

### *Preliminary screening of plant extracts*

Only the motility of adult worms was examined initially. Tables 2 and 3 summarize changes in the motility score of females and males cultured with extracts of various Guatemalan plants for 7 days. Untreated adult female and male worms remained highly active throughout the experiment. Of 12 extracts used, ten extracts examined reduced motility of both male and female adult worms. The remaining two extracts did not affect the motility of females. *Rauvolfia tetraphylla* and *Neurolaena lobata* caused both males and females to be completely immotile after 24 h incubation, although the concentration of *N. lobata* was lower than that of *R. tetraphylla*.

*Acalypha guatemalensis* and *Gliricidia sepium* also completely immobilized males and females but they took a longer time; *A. guatemalensis* taking 3 days for males and 4 days for females and *G. sepium* taking 4 days for males and 6 days for females.

### *Effects of* N. lobata *extracts*

*Motility and MTT assay*

Untreated adult female and male worms remained highly active throughout the experiments (fig. 1). The effect of *N. lobata* extracts on the motility of both female and male worms was observed in concentration- and time-dependent manner. Concentrations higher than 100 $\mu$g ml$^{-1}$ ceased motility of both sexes of adult worms. The time required to stop the motility of adult worms was 6 h at 500 $\mu$g ml$^{-1}$, 24 h at 250 $\mu$g ml$^{-1}$, and 3 days for females and 4 days for males at 100 $\mu$g ml$^{-1}$. A concentration of 50 $\mu$g ml$^{-1}$ stopped motility of females at 4 days but only reduced the motility of males. The lowest concentration of 10 $\mu$g ml$^{-1}$ reduced the movement of both sexes with a stronger activity against females than males.

The absorbance of formazan in DMSO per length of females and males after culturing with each concentration for days is shown in fig. 2. The MTT assay showed that the viability of female worms exposed to concentrations

Table 2. Changes in the motility score of adult male worms of *Brugia pahangi* cultured with various extracts of medicinal plants.

| Medicinal plants used | Concentrations tested ($\mu$g ml$^{-1}$) | Days of culture | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| Control | | 3.00 | 3.00 | 3.00 | 3.00 | 3.00 | 3.00 | 2.25 | 2.25 |
| *Tridax procumbens* | 1000 | 3.00 | 3.00 | 2.50 | 1.50 | 0.25 | 0.00 | 0.00 | 0.00 |
| *Acalypha guatemalensis* | 1000 | 3.00 | 3.00 | 0.25 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| *Croton guatemalensis* (1) | 250 | 3.00 | 3.00 | 3.00 | 3.00 | 3.00 | 1.75 | 1.25 | 1.25 |
| *Croton guatemalensis* (2) | 125 | 3.00 | 2.75 | 3.00 | 2.25 | 1.25 | 0.50 | 0.75 | 0.25 |
| *Chrysophyllum cainito* | 250 | 3.00 | 2.75 | 2.75 | 2.50 | 1.75 | 1.75 | 1.00 | 0.50 |
| *Petiveria alliacea* | 125 | 3.00 | 3.00 | 3.00 | 2.50 | 1.75 | 1.50 | 0.50 | 0.25 |
| *Smilax regellii* | 167 | 3.00 | 3.00 | 3.00 | 3.00 | 3.00 | 2.50 | 1.50 | 1.25 |
| *Tecoma stans* | 500 | 3.00 | 3.00 | 2.75 | 1.75 | 1.25 | 1.25 | 0.00 | 0.00 |
| *Psidium guajava* | 250 | 3.00 | 2.50 | 2.75 | 1.75 | 0.25 | 0.00 | 0.00 | 0.00 |
| *Gliricidia sepium* | 1000 | 3.00 | 2.50 | 2.00 | 1.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| *Rauvolfia tetraphylla* | 1000 | 3.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| *Neurolaena lobata* | 500 | 3.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |

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Table 3. Changes in the motility score of adult female worms of *Brugia pahangi* cultured with various extracts of medicinal plants.

| Medicinal plants used | Concentrations tested ($\mu g\,ml^{-1}$) | Days of culture | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| Control | | 3.00 | 3.00 | 3.00 | 3.00 | 3.00 | 3.00 | 3.00 | 3.00 |
| *Tridax procumbens* | 1000 | 3.00 | 3.00 | 2.50 | 2.00 | 2.00 | 1.00 | 0.75 | 0.25 |
| *Acalypha guatemalensis* | 1000 | 3.00 | 3.00 | 3.00 | 1.50 | 0.00 | 0.00 | 0.00 | 0.00 |
| *Croton guatemalensis* (1) | 250 | 3.00 | 3.00 | 2.75 | 3.00 | 2.50 | 3.00 | 3.00 | 3.00 |
| *Croton guatemalensis* (2) | 125 | 3.00 | 2.75 | 3.00 | 3.00 | 3.00 | 3.00 | 1.67 | 0.00 |
| *Chrysophyllum cainito* | 250 | 3.00 | 2.75 | 3.00 | 3.00 | 3.00 | 2.25 | 2.00 | 2.25 |
| *Petiveria alliacea* | 125 | 3.00 | 3.00 | 3.00 | 3.00 | 3.00 | 2.25 | 2.25 | 1.75 |
| *Smilax regellii* | 167 | 3.00 | 3.00 | 3.00 | 3.00 | 3.00 | 3.00 | 3.00 | 3.00 |
| *Tecoma stans* | 500 | 3.00 | 3.00 | 2.00 | 2.00 | 2.00 | 1.75 | 1.50 | 1.25 |
| *Psidium guajava* | 250 | 3.00 | 2.50 | 2.00 | 2.25 | 1.75 | 1.50 | 1.00 | 0.25 |
| *Gliricidia sepium* | 1000 | 3.00 | 2.50 | 1.00 | 2.00 | 0.75 | 0.25 | 0.00 | 0.00 |
| *Rauvolfia tetraphylla* | 1000 | 3.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| *Neurolaena lobata* | 500 | 3.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |

higher than 50 $\mu g\,ml^{-1}$ and the viability of males exposed to concentrations greater than 100 $\mu g\,ml^{-1}$ was significantly lower than that of control worms and comparable to that of heat-killed worms, indicating that these worms were dead.

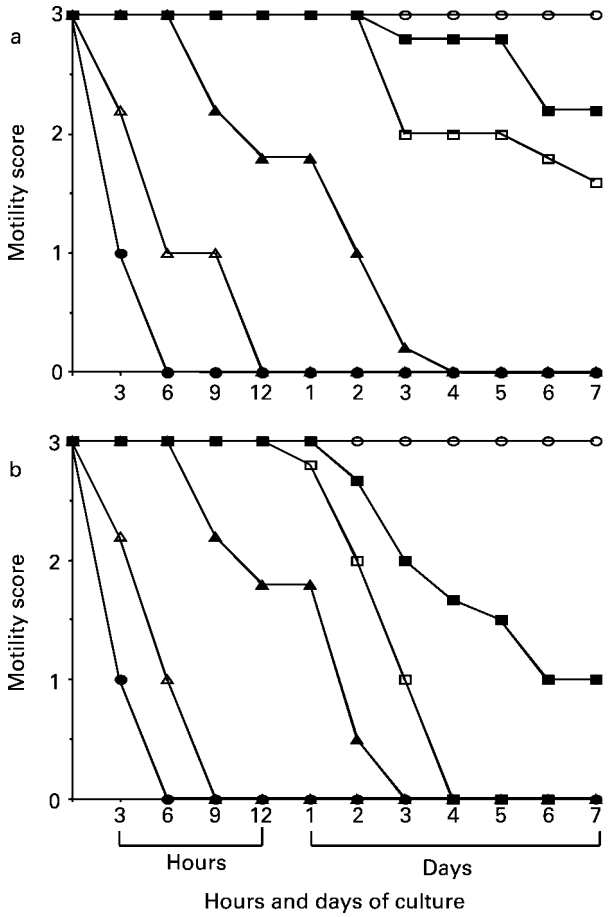


Fig. 1. Changes in the motility of males (a) and females (b) of *Brugia pahangi* exposed to ethanol extracts of *Neurolaena lobata*. ○, control; ●, 500 $\mu g\,ml^{-1}$; △, 250 $\mu g\,ml^{-1}$; ▲, 100 $\mu g\,ml^{-1}$; □, 50 $\mu g\,ml^{-1}$; ■, 10 $\mu g\,ml^{-1}$.

*Microfilarial release*

Changes in the number of microfilariae released by females cultured with various concentrations of *N. lobata* extract are shown in fig. 3. Females of untreated control groups showed a relatively stable microfilarial release throughout, with 86.0–130.5% of the original number of released on day 0. Compared with controls, all concentrations tested stopped the release during the 7-day incubation period in a concentration- and time-dependent manner, i.e. in 24 h by 500 and 250 $\mu g\,ml^{-1}$, in 2 days by 100 and 50 $\mu g\,ml^{-1}$, and in 5 days by 10 $\mu g\,ml^{-1}$.

*Microfilarial survival*

The survival rate of microfilariae exposed to various concentrations of *N. lobata* extract is shown in fig. 4. Untreated microfilariae showed a long survival rate for the first 3 days but afterwards the rate gradually lowered

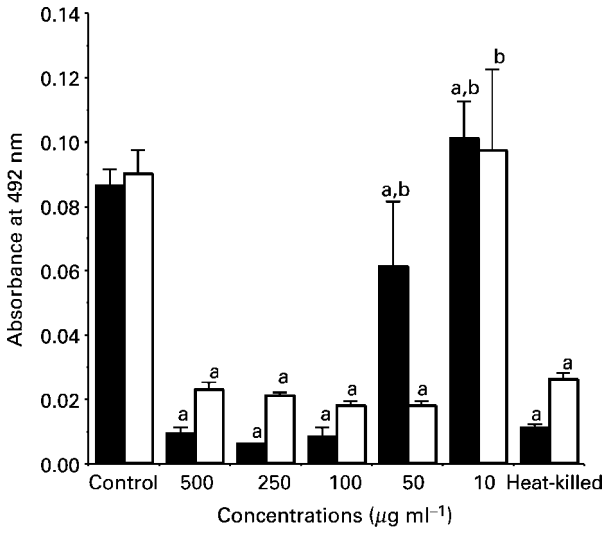


Fig. 2. Absorbance of formazan in DMSO per length (cm) at 492 nm of *Brugia pahangi* males (■) and females (□) cultured with ethanol extracts of *Neurolaena lobata* for 7 days. Letters, a and b above each bar represent significant differences from control and from heat-killed worms, respectively (Scheffe's test, $P < 0.05$).

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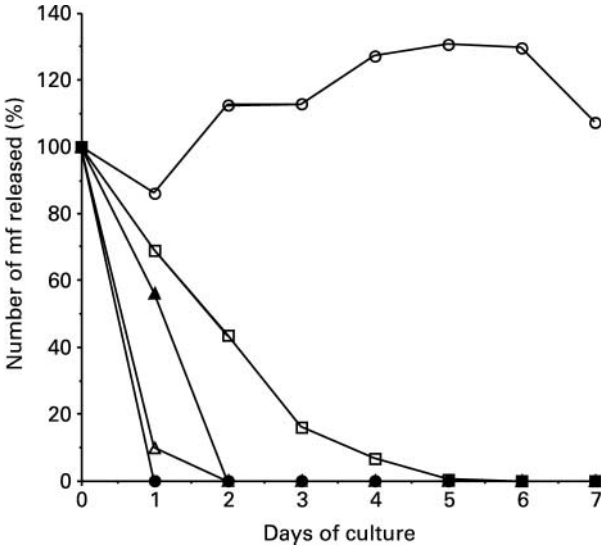


Fig. 3. Changes in the number of mircofilariae released by female *Brugia pahangi* cultured with ethanol extract of *Neurolaena lobata*. ○, control; ●, 500 and 250 $\mu g\,ml^{-1}$; △, 100 $\mu g\,ml^{-1}$; ▲, 50 $\mu g\,ml^{-1}$; □, 10 $\mu g\,ml^{-1}$.

until all microfilariae showed no movement after 7 days of culture. Compared with controls, concentrations higher than 100 $\mu g\,ml^{-1}$ killed microfilariae in a concentration- and time-dependent manner, i.e. 24 h by 500 and 250 $\mu g\,ml^{-1}$ and in 3 days by 100 $\mu g\,ml^{-1}$.

## Discussion

The plants used in the present study have been traditionally used by the local people in Guatemala. Firstly, the antifilarial potencies of 12 extracts from 11 medicinal plants were investigated. The assay system used was that described by Khunkitti *et al.* (2000). The motility of adult worms and the survival rate of microfilariae in control groups showed similar patterns to those obtained by Khunkitti *et al.* (2000). Of several extracts, a direct macrofilaricidal effect was observed in initial experiments although concentrations of the extracts tested were different. The strongest activity was observed in the ethanol extract of *N. lobata*. The osmolarity and pH of the culture medium are not thought to affect the motility of worms, as the osmolarity (301–304 mOsmol $kg^{-1}$) of the culture medium containing any extracts initially tested was comparable to that of control media (301 mOsmol $kg^{-1}$) and the pH of all media used was 7.4.

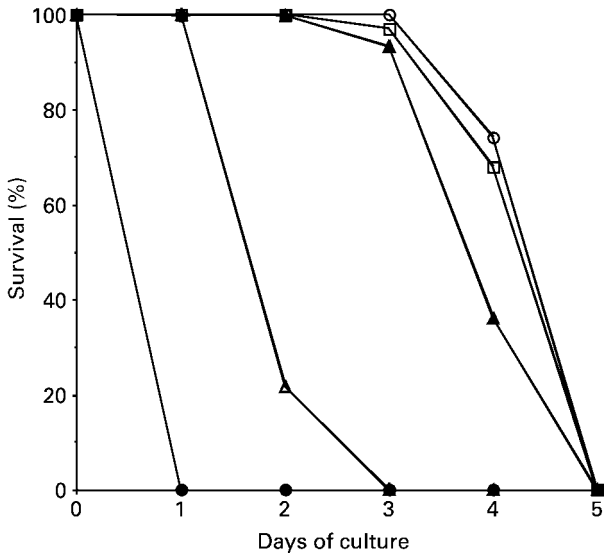


Fig. 4. Survival rates of microfilariae of *Brugia pahangi* exposed to ethanol extracts of *Neurolaena lobata*. ○, control; ●, 500 and 250 $\mu g\,ml^{-1}$; △, 100 $\mu g\,ml^{-1}$; ▲, 50 $\mu g\,ml^{-1}$; □, 10 $\mu g\,ml^{-1}$.

*Neurolaena lobata* is a herb widely used by the Guatemalan population to cure diseases causing dysentery (amoebiasis), fever with chills (malaria) and diabetes (Gupta *et al.*, 1984; Girón *et al.*, 1991). Ethanol extracts of the plant, which has been extensively studied for potential activity against bacteria, fungi and protozoa, have been found to be effective against these agents (Cáceres *et al.*, 1998).

The present study clearly demonstrated that the ethanol extract of leaves of *N. lobata* possessed macro- and micro-filaricidal effects against the filarial nematode *Brugia pahangi*. The effect was observed even at a low concentration of 50 $\mu g\,ml^{-1}$. Although several *in vitro* investigations of plant extracts have been reported, Comley *et al.* (1989) suggested that extracts of the stem bark of *Pachypodanthium staudtii* and *Streblus asper* may be macrofilaricidal. Macrofilaricidal effects of *S. asper* extracts were observed at 10 $\mu g\,ml^{-1}$ after 5 days incubation, which is comparable to the concentration of 50 $\mu g\,ml^{-1}$ showing macrofilaricidal effect in the present study.

Comley (1990) suggested that an early assessment of the comparative *in vitro* mammalian cytotoxicity of plant extracts should be undertaken prior to *in vivo* studies. The ethanol extract has previously been proved to have no toxicity to *Artemia salina* nauplii (brine shrimp) (Cáceres *et al.*, 1998). In mice the extract showed no oral acute toxicity neither did it show either oral or intraperitoneal subacute toxicity (Cáceres *et al.*, 1998).

The present study suggests that *N. lobata* may produce a promising macrofilaricidal drug. For a better understanding of the efficacy and mechanism of the action and for further development of new filaricides, *in vivo* studies using ethanol extracts of this Guatemalan medicinal plant are needed, as recommended by the WHO (1984, 1993).

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