Original Article

Antifilarial activity of gum from *Moringa oleifera* Lam. on human lymphatic filaria *Brugia malayi*

Abstract

Aim: Currently available antifilarial drugs diethylcarbamazine, ivermectin and albendazole and their combinations, are not able to control lymphatic filariasis. Therefore, a better antifilarial agent is urgently required for proper management of the disease. **Materials and Methods:** In this study, we evaluated the antifilarial activity of gum extract of plant *Moringa oleifera* Lam. against the human lymphatic filarial parasite *Brugia malayi* using adult worms and microfilariae (mf) in two *in vitro* assays (motility and inhition in MTT reduction) for viability and two animal models, primary (*Meriones unguiculatus* implanted with *B. malayi* adult worms in the peritoneal cavity) and secondary (subcutaneous *B. malayi* infective larvae induced *Mastomys coucha*, the model closer to the natural human filarial infection) screens. **Results:** The gum extract inhibited 100% motility (irreversible loss of motility) of mf and inhibited more than 56% MTT reduction potential of the adult female worms. The extract was safe in cytotoxicity test using Vero cell line, therefore followed *in vivo* in primary and secondary screens. In primary screen, the extract (5×500 mg/kg) caused 69% macrofilaricidal and 83% sterilization of female worms and 44% macrofilaricidal activity in secondary screen (5 × 1000 mg/kg) by oral route. **Conclusion:** Thus, it is concluded that the gum of the plant is macrofilaricidal in both *in vitro* and *in vivo* and may provide valuable leads for design and development of new antifilarial agents. This is the first ever report on the antifilarial efficacy of *M. oleifera*.

Key words:
Brugia malayi, lymphatic filariasis, Mastomys coucha, Meriones unguiculatus, Moringa oleifera, motility assay, MTT assay

Introduction

Human lymphatic filariasis (LF) caused by the nematode parasites *Brugia malayi*, *B. timori*, and *Wuchereria bancrofti* affects 120 million people worldwide, of which 40 million people show the chronic disease manifestations: Elephantiasis and hydrocele[1] which cause permanent and long-term disability.[2] About 1 billion people (18% of the world's population) are at risk of infection (www.globalnetwork.org). World Health Organization has recognized this major health problem and launched a global programme for elimination of filariasis[3,4] using yearly doses of multidrug regimen in a mass drug administration program for at least 5 years[5] to interrupt transmission and reduce morbidity. However, several technical challenges threaten the success of such eradication programs[2,6,7] including limited efficacy of available drugs diethylcarbamazine (DEC) and ivermectin against adult filarial worms. This depressing perspective demands an urgent need for an agent that kills and/or sterilizes the adult worms, because adult parasites not only produce millions of microfilariae (mf) that are picked up by mosquito vector and transmitted but also are responsible for the debilitating pathological lesions.

Since ages, several medicinal agents have been derived and developed from plants and used in traditional medical systems, such as Ayurveda, Unani, Siddha, and others. Recently, Murthy *et al*.[8] reviewed the contribution of plants

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in the prevention and treatment of LF and helminthic infections. Unfortunately, no plant could generate a success story in the management of LF, and therefore, the quest for an antifilarial plant continues. *Moringa oleifera*, or the horseradish tree, is a pan-tropical species known by regional names as benzolive, drumstick tree, kelor, marango, mlonge, mulangay, nébéday, saijhan, and sajna. All parts of the *Moringa* tree have long been consumed by humans and are considered to possess nutritional and medicinal properties.[9] Many parts of the plant *M. oleifera* Lam. were reported to possess anti-inflammatory, anti-oxidant, anti-microbial, anti-hyperlipidemic, anti-fertility, anti-cancer, anti-hepatotoxic, and anti-ulcer activities.[9,10] The resin/gum of the plant is reported to be useful as analgesic, antipyretic, and in the treatment of dysentery, asthma, syphilis, rheumatism, skin disorders, and in wound healing.[11-13] To the best of our knowledge, there are no studies on the use of the plant or its gum in the management of LF. This study was therefore undertaken to find out whether the gum of the plant has any antifilarial efficacy against human lymphatic filarial infection, especially against the adult worms.

Materials and Methods

Plant

M. oleifera Lam. was identified, authenticated (voucher specimen number 6399), and provided by the Botany Division, CSIR- Central Drug Research Institute (CDRI), Lucknow, India. Gum of the plant used in this study was collected from Lucknow and adjacent areas (and stored at 4°C) was provided by CDRI.

Extraction of gum

The gum was soaked for 24 hours at room temperature in 10 ml distilled water adjusted to pH. 7.0 stirring occasionally. After 24 hours, the supernatant was decanted and the gum was again soaked in fresh distilled water and stirred occasionally and the supernatant was collected after 24 hours. The supernatants were pooled and concentrated at reduced pressure below 45°C using a rotavapor. The material so obtained was stored at 4°C till use.

Bioactivity of the extract

Parasites

The human filarial parasite *B. malayi* was maintained in *Mastomys coucha*[14] and jirds (*Meriones unguiculatus*) as described elsewhere.[15] Mf and adult worms freshly harvested from the peritoneal cavity (PC) of the jirds exposed to infective third stage larvae (L_3) 5–6 months back were washed thoroughly in HBSS and used for *in vitro* assays and transplantation into PC of the jirds.

In vitro assays

The dried gum extract and reference compound DEC were dissolved in DMSO. The final concentration of DMSO in the incubation medium was always kept below 0.1%. The reference drug used was DEC. DMSO was used in place of test agent solution for controls. The parasite stages were incubated in Hanks Balanced Salt Solution (HBSS; Sigma, USA) at pH. 7.2 containing mixture of antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin; Sigma) for 24 hours at 37°C in 5% CO_2 atmosphere.

Initially, efficacy of the extract was assessed *in vitro* on mf and adult female worms broadly following the methods motility and or 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) reduction assays.[16,17] In brief, mf and female worms were isolated from PC of jirds harboring 4–5 months old *B. malayi* infection.[14,15] In the first set of experiment, the concentrations of the extract and DEC were used at 1000 μg/ml in the assay systems. After exposure to the agents, the effect on motility of both mf and adult worm was assessed (viability as end point); the same treated and control adult female worms were processed for assessing percentage inhibition in the MTT reduction. Extract showing effect on motility, i.e., irreversible loss of motility (100% inhibition in motility) and 50% or more inhibition in MTT reduction were considered active and subsequently subjected for secondary *in vitro* screening for assessing IC50 value. For assessing IC50, two-fold dilutions of the extract and DEC were used starting from 1.95 to 1000 μg/ml using triplicates of 40–50 mf/100 μl/well in 96-well plate and one adult female worm/ml/well of 48-well plate (Nunc, Denmark).

Assessment of in vitro efficacy

The viability of the treated worms was assessed by calculating percent inhibition in motility and MTT reduction over DMSO-treated worms.[16,17] Parasite motility was assessed under a microscope after 24 hours exposure to test substance and scored as: 0 = dead; 1–4 = loss of motility (1 = 75%, 2 = 50%, 3 = 25%, and 4 = no loss of motility). Loss of motility is defined as the inability of the worms to regain pretreatment level of motility even after incubating in fresh medium *minus* the test agent at 37°C for 25–30 min and is expressed as percentage (%) inhibition of control. 100% inhibition in motility of female adults or mf and or ≥50 inhibition in MTT reduction ability of adult parasites was considered acceptable antifilarial (microfilaricidal/adulticidal) activity.

Determination of IC50 and cytotoxic concentration 50 (CC50)

IC50 (the concentration at which the parasite motility was inhibited by 50%) of the extract and DEC was determined as described in detail by Lakshmi *et al*.[17]

The cell cytotoxicity assay of the test substances using VERO Cell line C1008 (African green monkey kidney cells) in DMEM supplemented with 10% heat inactivated FBS was carried out following the method of Pagé *et al*.[18] with some modifications.[17,19] Briefly, VERO Cell line C1008 was

seeded in 96-well microtiter plates (Nunc) at 0.1 × 10^6 cells/ml (100 µl per well) in DMEM supplemented with 10% heat-inactivated FBS. A three-fold serial dilution of the test substances starting from >20 × LC100 concentration of the test agent was added in the medium and incubated for 72 hours at 37°C in 5% CO_2 atmosphere followed by addition of Resazurin as viability indicator.[17] Data obtained from IC50 and CC50 assays were transferred into a graphic program (MS Excel) and calculated by linear interpolation between the two concentrations above and below 50% inhibition.[20] The assay was run in replicates in each of the two independent experiments.

Selectivity index (SI) of the agents were computed by the formula SI = CC50/IC50.

In vivo efficacy

Host-parasite models

For evaluation of efficacy of the extract against *B. malayi*, two models *M. coucha* and *M. unguiculatus* infected with *B. malayi* were used. *M. unguiculatus* implanted with *B. malayi* adult worms in the PC served as primary model. Infection initiated by subcutaneous injection of L_3 of *B. malayi* in *M. coucha* was used as secondary model (which is closer to the natural human lymphatic filarial infection mode).

Healthy young adult 6–8 weeks old *M. coucha* and *M. unguiculatus* were obtained from National Laboratory Animal Center, CDRI, Lucknow, and maintained throughout the study, in climate (23±2°C; RH: 60%) and photoperiod (12-hour light-dark cycles) controlled animal quarters. They were fed standard rodent chow supplemented with dried shrimps (*M. coucha*) and had free access to drinking water.

All the experiments were conducted in compliance with the Institutional Animal Ethics Committee guidelines for use and handling of animals. At the end of observation period, the treated and control animals were autopsied following the guidelines of Institutional Animal Ethics Committee.

For establishment of transplantation-induced infection in jirds, the method of Sadanaga *et al*.[21] was adopted with some modifications to suit our conditions.[22] Briefly, 8–10 weeks old male jirds were transplanted intraperitoneally (i.p.) with adult worms isolated from the PC of jirds harboring 4–5 months old *B. malayi* infection. Each jird received 10 female and 5 male worms. On day 2 or 3 of the adult worm transplantation, the peritoneal fluid was aspirated and checked for the presence of mf. The treatment was initiated on day 7 or 8 post transplantation (p.tpl.) as per the method of Sadanaga *et al*.[21]

B. malayi infection in male *M. coucha* was produced by inoculating 100 L_3 obtained from experimentally infected laboratory-bred black eyed susceptible strain of *Aedes aegypti* mosquitoes which were fed on microfilaremic *M. coucha* 9/10 days before as described by Murthy *et al*.[14]

Selection of *M. unguiculatus/M. coucha-B. malayi*

A total of 20 transplantation-induced jirds were used for the study. Four animals were treated with the gum extract and an equal number of animals were kept as untreated controls. DEC-C (standard drug) treated and untreated control groups consisted of six animals each.

M. coucha harboring about 5–6 months old infection and showing progressive increase in mf counts were selected for the study. Six to seven animals each were included for treated and untreated infected groups.

We carried out animal experiments with the extract and DEC at different time points, each time with their respective concurrent controls and analyzed the results comparing with their respective controls only and not with control from other time point.

The animals were killed under deep ether anesthesia.

Administration of extract and standard drug DEC

For animal feeding, the gum extract was prepared in 1% gum acacia in triple distilled water and was tested at 500 mg/kg and 1 gm/kg for 5 days, respectively, against *B. malayi* in jird and *M. coucha*. The treatment was given through oral route.

DEC-citrate (DEC-C) was used as the control drug under similar conditions against the infections at recommended standard doses (25 mg/kg in jird and 50 mg/kg in *M. coucha*) orally. Both extract and standard drug were given for 5 consecutive days.

Sex- and age-matched identically infected untreated animals were administered with vehicle only and kept as controls.

Assessment of antifilarial activity in primary screen (*B. malayi*-jird)

Microfilaricidal efficacy was assessed in peritoneal aspirate on day 7/8 post initiation of treatment (p.i.t.) and on autopsy macrofilaricidal activity was determined on day 56/60 p.i.t.

Assessment of antifilarial activity in secondary screen (*B. malayi-M. coucha*)

Micro- and macrofilaricidal efficacy of the extract and DEC was assessed according to the method of Lammler and Wolf[23] with some modifications.[24] Ten cubic millimeters of tail blood of treated and control animals were examined just before treatment and thereafter at weekly intervals till day 91 p.i.t. The intensity of microfilaricidal activity as percent change in population of mf over pretreatment levels was calculated.

For assessment of macrofilaricidal activity, the treated and control animals were killed on day 91 p.i.t. Adult male and

female worms were isolated from p.c. (in case of jirds) and various tissues (in case of *M. coucha*) of the animals, kept in normal saline and examined under microscope for motility and cell adhesion on the surface of the worm. The percent mortality of adult filariids was calculated by comparing the live worms recovered from treated animals with that of the total number of live worms recovered from untreated (control) animals. All surviving females were teased individually in saline on glass slides, and the conditions of mf and its developing embryonic forms in uteri were examined under microscope. Female worms with empty uteri or uteri containing dead or degenerated embryos or mf were considered sterile worms. Percent sterility in live female worms recovered from the treated and control animals were calculated over total live female worms recovered.

Statistical analysis

Results were presented as mean ± S.D. of —four to seven animals in two independent experiments and the data were analyzed statistically by Student's *t test* using GraphPad Prism®. Differences with $P<0.05$ were considered significant.

Results and Discussion

***In vitro* efficacy**

The extract of gum of the plant was screened for antifilarial activity against mf and adults (macrofilariae) of *B. malayi in vitro*. In motility assay, the extract killed mf at 125 μg/ml but not adult worms. However, it inhibited 56% MTT reduction potential of the adult worms at 1000 μg/ml [Table 1]. The gum extract was safe in cytotoxicity study *in vitro*. In summary, the findings show that the gum extract could affect both mf and adult worms *in vitro* and is non-toxic.

***In vivo* efficacy**

The gum extract was tested at 500 mg/kg, p.o. for 5 days in *B. malayi*/jird model. It was ineffective against mf of PC of animals on days 7/8 p.i.t. but produced significant ($P<0.01$) adulticidal (around 69%) and female sterilization (83%) effect [Table 2]. DEC-C at 25 mg/kg, (p.o. for 5 days) did not produce any antifilarial activity. Untreated control animals showed live and motile worms [Table 2] with 13–14% sterile female worms.

Against *B. malayi* in *M. coucha*, the extract of gum administered at 1000 mg/kg, p.o. for 5 days caused 44% adulticidal and poor sterilizing effect on female worms. The extract did not exert any significant effect on mf on day 7/8 p.i.t. as compared with pretreatment level, but the level of microfilaremia remained almost same till killing of the animals [Table 3].

In summary, the extract showed significant macrofilaricidal activity in both the animal models.

As expected DEC-C at 50 mg/kg caused more than 83% ($P<0.001$) reduction in microfilaremia on day 7/8 p.i.t. in *B. malayi*/*M. coucha* model but affected 35% adult worms, and around 26% of surviving females was found sterilized.

Untreated control animals (*M. coucha*) showed progressive rise in microfilaraemia till termination of the experiment. Between 20 and 25% of live female worms recovered from these animals were found sterile [Table 3].

The extract apparently did not produce any adverse effect in general behavior of the jirds or *M. coucha* with *B. malayi*

Table 1: *In vitro* activity of *Moringa oleifera* on adult worms and microfilariae of *Brugia malayi*

Antifilarial agent	Effect on female adult worm			Effect on microfilariae (mf)		CC_{50}[c] (μg/ml)	SI w.r.t. mf motility
	LC100[a] (μg/ml) in motility assay	IC_{50} (μg/ml) in motility assay[b]	Mean % inhibition in MTT reduction	LC100 (μg/ml) in motility assay	IC_{50}/EC50 (μg/ml) in motility assay[b]		
Gum extract	>1000	>1000	56.15	125	74.33	5694.19	76.61
DEC-C	800	288.67	64.37	500	353.55	9102.85	31.88

[a]100% reduction in motility indicates death of parasite; [b]IC_{50} – 50% concentration of the agent at which 50% inhibition in motility is achieved; [c]CC_{50} – concentration at which 50% of cells are killed; SI – Selectivity index (CC_{50}/IC_{50}); DEC-C – Diethylcarbamazine-citrate

Table 2: Antifilarial activity of *Moringa oleifera* and DEC against transplanted adult worms of *Brugia malayi* in *Meriones unguiculatus*

Antifilarial agent	Dose, route for 5 days (no. of animals)	Effect on microfilariae in peritoneal cavity	No. of live worms			No. of sterilized female worms (%)
			Male	Female	Total[a]	
Gum extract	500 mg/kg, oral (4)	No effect	2.3±0.9	1.5±1.0	3.8±1.5 (69.1)**	83
Untreated control for gum extract	Vehicle, oral (4)	No effect	3.6±1.1	8.5±0.9	12.1±1.6	13
DEC-C	25 mg/kg, oral (6)	No effect	1.7±1.5	8.7±1.5	10.3±2.9 (14.8)	11
Untreated control for DEC-C	Vehicle (6)	No effect	3.5±1.3	9.0±0.8	12.5±1.9	14

Values are mean±SD; [a]% reduction in worm burden over control; DEC-C – Diethylcarbamazine-citrate; $^{**}P<0.01$ (vs control)

Table 3: Antifilarial activity of *Moringa oleifera* and DEC against L_3 induced *Brugia malayi* in *Mastomys coucha*

Antifilarial agent	Dose, route for 5 days (no. of animals)	Microfilariae in 10 μl blood at days post initiation of treatment			No. of live worms			No. of sterilized female worms (%)
		Day 0	Day 7/8	Day 90	Male	Female	Total[a]	
Gum extract	1000 mg/kg, oral (7)	78.3±33.2	102.3±69.6	100.2±24.0	4.0±2.7	13.7±3.5	17.7±5.7 (43.6)**	2.3±1.2 (17)
Untreated control for gum extract	Vehicle, oral (6)	53.5±31.7	113.67±52.6	169.80±64.2	6.3±3.8	25.0±10.0	31.3±13.6	6.3±4.5 (25)
DEC-C	50 mg/kg, oral (6)	72.2±23.9	15.7±5.6	50.7±17.8	6.00±1.8	14.3±4.0	20.3±3.5	3.7±6.1 (26)
Untreated control for DEC-C	Vehicle, oral (7)	51.3±29.4	106.7±51.5	168.2±57.5	7.00±3.5	12.5±3.0	19.5±3.0	2.5±1.4 (20)

Values are mean±SD; DEC-C – Diethylcarbamazine-citrate; **$P<0.01$ (vs control)

during the 5-day treatment and thereafter till the day of killing (56/60 days in case of jirds and 91 days in case of *M. coucha*).

Globally, the disease is second to malaria in causing permanent and long-term disability disease of the limbs and genitals, resulting not only in physical crippling but also in serious psychosocial consequences.[3] The available antifilarial efficacies have inherent limitations. The adult filarial worms can survive in the human host for more than 6 years or so and therefore, current treatment with microfilaricides such as DEC and ivermectin are given either in combination with albendazole or alone. Drug resistance may become a critical issue after prolonged mass treatment with the current drugs, especially with ivermectin in Africa where DEC cannot be used. Therefore, we need new antifilarials for replacement of the existing antifilarial drugs. We are involved in the development of macrofilaricide or female worm sterilizing agents from natural products. The present investigation deals with the antifilarial efficacy of gum of the plant *M. oleifera* against lymphatic filarial infection.

The extract of gum was found effective against the microfilarial stage of the human lymphatic filarial parasite, *B. malayi in vitro*. However, the extract could inhibit the MTT reduction potential of the adult worms. Interestingly, in jird-*B. malayi* model it affected significant number of adult worm (killing of the worms and sterilization of female worms). In L3-induced *B. malayi* infection in *M. coucha* model which is closer to the natural human filarial infection, the extract caused significant macrofilaricidal activity. As reported earlier, DEC demonstrates strong microfilaricidal action against *B. malayi* in mastomys but is inactive in jird model.[25]

The mechanism by which the gum extract exerts its filaricidal activity *in vitro* and *in vivo* is not known. It contains L-arabinose, D-galactose, D-glucuronic acid, L-rhamnose, D-mannose, and D-xylose. Besides these compounds, a leucoanthocyanin characterized as leucodelphinidin-3-O-B-D-galactopuranosy (1- >4)-O-B-D-glucopyranoside is also present in *M. oleifera*.[26] Recent studies have shown that *M. oleifera* has anti-helminthic, antibiotic, detoxifying, immune building activities, and also is used to treat malaria.[27,28] Most filarial parasites have symbiont bacteria *Wolbachia* which is known to confer survival value to the parasites.[29] It was reported that plant products rich in anthocyanins such as *Hisbiscus sabdariffa*[17,30] exert macrofilaricidal effect via elimination of *Wolbachia*. This could be a possible mechanism through which the gum extract might have affected because it is also rich in leucoanthocyanidines. Currently, we are carrying out further studies in this direction.

It is concluded that the gum of the plant in macrofilaricidal in both *in vitro* and *in vivo* and may provide valuable leads for design and development of new antifilarial agents. This is the first ever report on the antifilarial efficacy of *M. oleifera* against experimental human *B. malayi* infection.

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