

Gedunin and photogedunin of *Xylocarpus granatum* possess antifilarial activity against human lymphatic filarial parasite *Brugia malayi* in experimental rodent host

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Abstract The present study is aimed to evaluate antifilarial activity of *Xylocarpus granatum* (fruit from Andaman) against human lymphatic filarial parasite *Brugia malayi* in vivo. The in vitro antifilarial activity has already been reported earlier for this mangrove plant which has traditionally been used against several ailments. Aqueous ethanolic crude extract, four fractions (ethyl acetate fraction, *n*-butanol fraction, water-soluble fraction and water-insoluble fraction) and pure molecule/s of *X. granatum* (fruit) were tested in vitro on adult worms and microfilariae (mf) of *B. malayi* and the active samples were further evaluated in vivo in *B. malayi* (intraperitoneally) i.p. transplanted in the jird model (*Meriones unguiculatus*) and *Mastomys coucha* subcutaneously infected with infective larvae (L3). The crude aqueous ethanolic extract was active in vitro (IC₅₀: adult=15.46 µg/ml; mf=13.17 µg/ml) and demonstrated 52.8% and 62.7% adulticidal and embryostatic effect on *B. malayi*, respectively, in *Mastomys* at a dose of 5×50 mg/kg by oral route. The antifilarial activity was primarily localized in the ethyl acetate-soluble fraction which revealed IC₅₀ of 8.5 and 6.9 µg/ml in adult and mf, respectively. This fraction

possessed moderate adulticidal and embryostatic action in vivo in *Mastomys*. Out of eight pure molecules isolated from the active fraction, two compounds gedunin (IC₅₀=0.239 µg/ml, CC₅₀=212.5 µg/ml, SI=889.1) and photogedunin (IC₅₀=0.213 µg/ml, CC₅₀=262.3 µg/ml, SI=1231.4) at 5×100 mg/kg by subcutaneous route revealed excellent adulticidal efficacy resulting in to the death of 80% and 70% transplanted adult *B. malayi* in the peritoneal cavity of jirds respectively in addition to noticeable microfilaricidal action on the day of autopsy. The findings reveal that the extract from the fruit *X. granatum* contains promising in vitro and in vivo antifilarial activity against human lymphatic filarial parasite *B. malayi* which could be attributed to the presence of two pure compounds gedunin and photogedunin.

Introduction

Human lymphatic filariasis a vector-borne disease mainly caused by *Wuchereria bancrofti*, *Brugia malayi* and *Brugia timori* continues to cause morbidity in the tropical and subtropical population. *W. bancrofti*, the predominant filarial parasite, affects more than 90% of lymphatic filarial patients, causing acute and chronic morbidity (Ottesen 2000). The activity of mainstay antifilarial drugs diethyl-carbamazine and ivermectin in combination with albendazole or alone is largely restricted to microfilaricidal activity. Threat of resistance to mainstay drugs is worrisome as the evidence is already revealed in various veterinary infections. Hence, there is an urgent need of new and potent drug which may either kill the adult parasite or adversely affect the reproductive potential of adult worms in addition to killing of microfilariae.

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The traditional systems of medicine provide an extremely vast body of source material for the development of new drugs and therefore have attracted researchers. Several natural products had earlier proved themselves against many species of filarial infections, e.g. *Andrographis paniculata* caused 100% mortality of *Dipetalonema reconditum* microfilariae, rhizome of *Zingiber officinale* (Zingiberaceae) reduced *Dirofilaria immitis* microfilariae load (Dutta and Sukul 1987), extract from the bark of *Streblus asper* was effective on chronic stages of filarial infection (Singh and Ram 1988), extracts prepared from *Carapa procera*, *Polyalthia suaveolens* and *Pachypodanthium staudtii* exhibited microfilaricidal activity on *Onchocerca volvulus* (Titanji et al. 1990). *Xylocarpus* also known as ‘dabi’ (or legi legi) belongs to the order Geraniales of the family Meliaceae. It is locally referred as the ‘puzzle nut tree’ (Alvi et al. 1991), and in folklore, this plant has been used as an astringent and febrifuge (Uddin et al. 2007). The genus *Xylocarpus* is distributed in the coastal regions of India, Ceylon, Burma and Malaya, is a large spreading mangrove, with rounded coriaceous leaves, smooth thin bark and abundant red heartwood, which furnishes a useful, timber of the characteristic mahogany type (Chopra et al. 1956). It occurs mainly in swamps on the ocean coast ranging between East Africa and Pacific islands, East Africa and Queensland, where it extends as far south as Cairns. The fruit is of grapefruit size, hard and heavy, leading to the common name ‘cannon ball tree’ (Mulholland and Taylor 1992; Lakshmi and Gupta 2008). Several pharmacological activities have been assigned to this plant viz. anti-diarrheal (Rouf et al. 2007), anti-cholera, antibacterial (Alam et al. 2006) anti-malarial (Omar et al. 2003), anti-pyretic and as an astringent and emollient (Yusuf et al. 1994). Seed ash is mixed with sulphur and coconut oil and applied as ointment for itch (Chopra et al. 1956). In vitro effect of crude aqueous extract from different parts of *X. granatum* on filarial parasite has been reported earlier (Zaridah et al. 2001). This prompted us to evaluate antifilarial activity in the aqueous ethanolic extract of *X. granatum* fruit both in vitro as well as in vivo using human lymphatic filarial parasite, *B. malayi* in two rodent models viz. jird and *Mastomys*. Several pure compounds were isolated and assessed for their in vitro activity on adult *B. malayi*. The active ones were followed in vivo for localizing the activity in the pure molecule/s.

Materials and methods

Plant material

X. granatum plant grows naturally in tidal forests along the East and West coastal areas up to Maharashtra and in Andaman Island. The fruits were collected in the month of

January from South Andaman Coast and were identified by the Division of Botany, Central Drug Research Institute (CDRI), Lucknow. The voucher of this specimen is kept at the herbarium of CDRI (acquisition number 328).

Extraction/fractionation procedure

Air-dried powdered fruits (1.0 kg) were extracted with 50% aqueous ethanol 5×5.0 L. and the combined extracts were filtered, concentrated under reduced pressure below 50°C to a minimum volume of 1.0 L. It was further dried in hot-air vacuum oven at 45°C to brown powder (yield 15%). The brown powder was further fractionated into chloroform-soluble (yield 8.5% of the 50% aqueous ethanol extract) and chloroform-insoluble fractions by maceration with chloroform. The chloroform action on repeated column chromatography over silica gel and final purification by HPLC on reverse phase C₁₈ R.P. columns using acetonitrile–water 55:45, v/v, flowrate of 1.0 ml/min using UV detector (λ , 230 nm) yielded compounds namely gedunin (36%) (Taylor 1974), photogedunin (2%) (Lakshmi et al. 2010) as shown in Fig. 1. All these were characterized using IR, NMR, mass, derivatization and comparing the data with those given in literature for these compounds. These were also compared with authentic samples on thin layer plates as well as their spectral data.

Infection

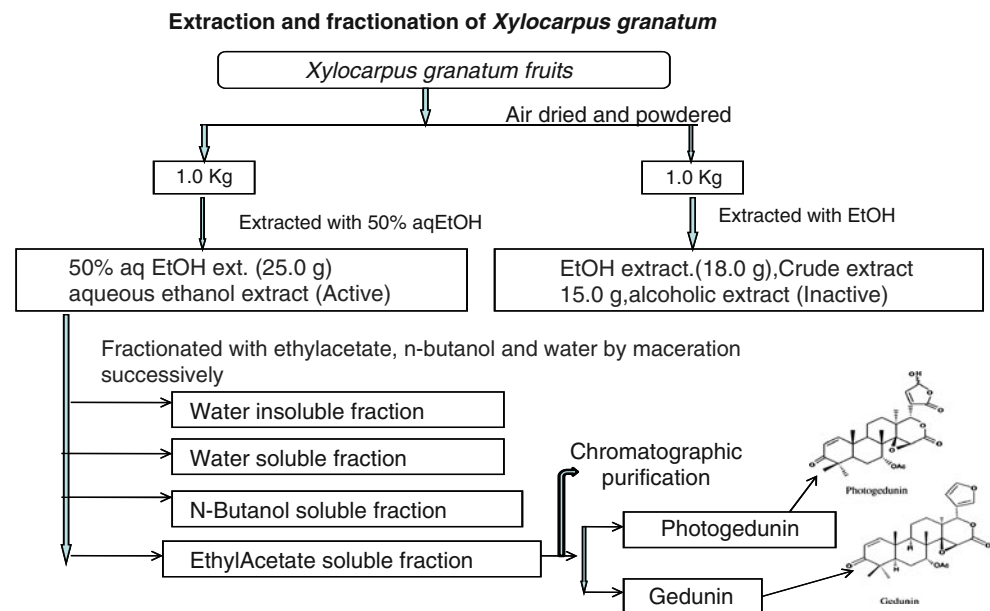
Sub-periodic strain of *B. malayi* was maintained in rodent hosts *Mastomys coucha* and *Meriones unguiculatus* (jird) through laboratory bred *Aedes aegypti*. Six-week-old male *Mastomys* were infected by subcutaneous inoculation of 100 infective larvae (L3) of *B. malayi* (Petranji et al. 1975) while jird received 150 L3 intraperitoneally (McCall et al. 1973). L3 were recovered from gently crushed mosquitoes by Baerman’s technique on day 9±1 post infective feeding on donor *Mastomys*.

Adult parasites for in vitro drug testing were recovered by washing the peritoneal cavity of jird infected 4 to 6 months back while in vivo testing of extracts was done in *Mastomys* infected 5 to 6 months back showing progressive rise in microfilaraemia. The identified pure compounds were further evaluated in vivo in jird intraperitoneally transplanted with adult *B. malayi*.

Antifilarial activity

In vitro efficacy

Sample preparation The stock suspension of plant samples and the standard drugs ivermectin as well as diethylcar-

Fig. 1 Extraction and fractionation of *X. granatum*

bamazine (DEC) (5 mg/ml) was prepared in DMSO for in vitro evaluation. The in vitro activity of the crude extract and fractions was assessed at serial twofold dilutions of the stock starting from 500 $\mu\text{g/ml}$ to the lowest concentration of 3.9 $\mu\text{g/ml}$. The pure compounds were tested at various twofold dilutions starting from 31.25 to 0.12 $\mu\text{g/ml}$.

Parasite isolation Adult worms and microfilariae (mf) of *B. malayi* were recovered aseptically from the peritoneal cavity of infected jirds within 120–150 days of intraperitoneal inoculation of 200–250 L3 of *B. malayi* (McCall et al. 1973). Microfilariae were isolated by passing the suspension through 5.0- μm membrane filter and thereafter pelleting.

In vitro testing The samples were tested at various concentrations on actively motile female worms in 48-well culture plate in duplicate containing 1,000 μl media with one female parasite/well (NUNC). RPMI 1640 medium containing antibiotics (penicillin 100 units/ml, streptomycin sulphate 100 $\mu\text{g/ml}$ and neomycin mixture; Sigma, USA) and fortified with 10% foetal bovine serum was used. The worms were exposed to test sample for 48 h at 37°C in CO₂ incubator. At the end of drug exposure, the motility of worms was recorded microscopically 1 h after transfer to fresh drug-free medium. The parasites were then processed for the 3-(4, 5 dimethylthiazol- 2-yl)-2, 5 diphenyl tetrazolium bromide (MTT) reduction assay as described earlier (Mukherjee et al. 1998). Activity of the sample was also assessed against mf (~20 live mf/well) using 96-well plate in duplicate containing 200 μl medium to which various twofold dilutions (125 to 0.12 $\mu\text{g/ml}$) of stock solution of compounds were added. The incubation conditions were the same as with adult parasite and mf motility was microscopically assessed.

Activity evaluation criterion The loss in motility of both adult female worm as well as mf and the percentage inhibition in MTT reduction in treated adult parasite compared to respective untreated controls were evaluated. Lethal concentrations (LC100) of adult *B. malayi* and mf were determined as the minimum concentration of test sample causing total irreversible immobility (death), the motility scoring of the adult worms as well as mf was done (0% motility reduction=4+; 1 to 49%=3+; 50 to 74%=2+; 75 to 99%=1+; 100%=D). Ivermectin was used as a standard filaricide (IC₅₀ adult 1.61 $\mu\text{g/ml}$ and IC₅₀ mf 3.62 $\mu\text{g/ml}$) for in vitro screen while DEC served as a standard filaricide for in vivo screen since it was inactive on both adult and mf in vitro.

The criterion for adulticidal activity was 100% irreversible immobility of adult worm with $\geq 50\%$ inhibition in MTT reduction by treated parasite over untreated control (Mukherjee et al. 1998).

Evaluation of IC₅₀ For assessing 50% inhibitory concentration (IC₅₀), further twofold dilutions of each test material was tested starting from LC100 (value up to 0.3 $\mu\text{g/ml}$). IC₅₀ concentration is determined by Excel-based line graphic template after plotting concentration values of each sample against percent inhibition in motility of parasite on x- and y-axis (Ramirez et al. 2007). Only the motility data was considered for evaluating IC₅₀ since inhibition in MTT reduction sometimes does not proportionately correlate with degree of motility reduction.

CC₅₀ evaluation In vitro cytotoxicity assay with all the test samples was carried out for assessment of CC₅₀ (50% cytotoxicity concentration) values following the method of O'Brien et al. (2000) with modifications. Vero cells

(monkey kidney cell line) in culture flask were trypsinized and counted using Neubauer chamber. Cell suspension/plate of 6.5 ml of (10^5 cells/ml) is required for assessing CC50 in a 96-well plate format. A minimum essential medium (MEM) of 100 μ l/well of was added to each third column of 96-well culture plate without vero cells to serve as negative control. One hundred microlitres of the above cell suspension is plated in to each well except the third, sixth, ninth and 12th column and incubated overnight at 37°C in CO₂ incubator. Medium was removed from all the wells and replaced by 100 μ l of fresh medium. One hundred microlitres of medium containing 300 μ g/ml test sample (highest concentration) was added to row H in triplicates (i.e. compound 1 from columns 1 to 3, compound 2 from columns 4 to 6, compound 3 from columns 7 to 9 and compound 4 from columns 10 to 12). Serial dilutions (3:1) were prepared with a multichannel pipette by transferring 50 μ l from row H to row G and mixing it and re-transferring 50 μ l in the same way to each consecutive row till row B. Row A was kept drug free as it serves as positive control and the plate was incubated for 48 h at 37°C in 5% CO₂ in air. After 72 h of drug exposure, 10 μ l/well of viability marker dye Resazurin or Alamar blue (stock solution 12.5 mg/100 ml PBS) was added and the plate was incubated for another 2–4 h. Fluorimetric reaction was measured using an excitation wavelength of 536 nm and an emission of 588 nm in a fluorimetric plate reader. Data was transferred to Excel and plotted as per the template using fluorescent signal against corresponding drug concentration. CC50 values were determined directly.

Determination of selectivity index To find out the safety of the test sample for further in vivo follow-up, selectivity index (SI) values of the in vitro active extracts was determined as the ratio of CC50 and IC50 ($SI = CC50/IC50$). A test substance showing SI value of ≥ 10 was considered safe for further into in vivo evaluation.

In vivo efficacy

Sample preparation A fine suspension of crude extract was prepared in distilled water; however, the pure compound was solubilised in distilled water with the help of 0.1% Tween-80.

Screening models

Adult *B. malayi* i.p. transplanted in jird Six- to eight-week-old male jird was intraperitoneally transplanted with ten females and five male adult worm of *B. malayi* recovered from jirds infected by L3 inoculation into the peritoneal

cavity. The worms were introduced into the recipients' peritoneal cavity by incising the abdomen under Ketamine anaesthesia (50 mg/kg, i.p.). On day 6/7, a drop of peritoneal fluid was aspirated and checked under the microscope for the presence of live mf to ensure successful transplant.

Subcutaneous *B. malayi* L3 infected *Mastomys coucha* *Mastomys* were infected by subcutaneous inoculation of ~100 infective larvae each recovered from *A. aegypti* fed on donor *Mastomys* 8/9 days back (Singh et al. 1997). Animals were monitored for microfilaraemia in 10 μ l tail blood between 1200 and 1300 hours from day 120 onwards and those infected 5–8 months back showing progressive rise in microfilaraemia were selected for the treatment.

Treatment schedule The crude extracts as well as fractions of *Xylocarpus granatum* were administered orally for five consecutive days at 250 mg/kg body weight, while the pure compounds were given at 100 mg/kg for five consecutive days by subcutaneous (s.c.) route in adult i.p. transplanted in the jird model. DEC was used as a standard drug and fed orally to *Mastomys* at 50 mg/kg body weight while in jird it was administered by s.c. route at 100 mg/kg.

Assessment of antifilarial activity

Subcutaneous *B. malayi* L3 inoculated *Mastomys model* Microfilaricidal as well as adulticidal (macrofilaricidal) activity of the crude extract was evaluated in *Mastomys* as described earlier (Misra-Bhattacharya et al. 2004). Thick blood smears of 10 μ l tail blood were made from treated and untreated animals just before starting the treatment, i.e. on day 0 and on days 8 and 15 after initiation of treatment thereafter at every fortnight till day 90. Percentage change in microfilaraemia was evaluated at each time point over pretreatment level and denoted as microfilaricidal efficacy. Animals infected under identical conditions received only vehicle to serve as controls. At the end of the observation period (on day 91), the treated and control *Mastomys* were euthanized and various tissues (lungs, heart, testes, lymph nodes) were teased gently in phosphate-buffered saline (PBS, pH 7.2) to recover the adult parasites. Worms were examined for their motility, death or encapsulation and the female worms were observed for their uterine contents as in case of *Mastomys* to assess the embryostatic effect.

I.P. adult *B. malayi* transplanted jird model At the end of the observation period (on day 50), the treated and control jirds were euthanized and worms were recovered from peritoneal cavity. Worms were teased gently in phosphate-buffered saline (PBS, pH 7.2) to recover adult parasites

(Misra-Bhattacharya et al. 2004). These were examined for their motility, death or encapsulation. All surviving females were teased individually in a drop of saline and the condition of the embryonic stages in the uteri was examined microscopically. Any abnormality or death/distortion detected in the uterine contents, including oocytes, eggs and mf were considered as a sterilization effect of the extract on the female and a percentage of sterile females were assessed (Misra et al. 1984).

Animal groups For crude extract and ethyl acetate-soluble fraction, three infected *Mastomys* were included in two experiments each; for other fractions, single experiment was carried out with only three *Mastomys* (experiment not repeated due to inferior activity); while for standard drug DEC, only five *Mastomys* were used in a single experiment. Control *Mastomys* group receiving vehicle consisted of five animals each in duplicate experiments.

Regarding jirds, three i.p. transplanted in jirds in each duplicate experiment were employed for each test sample.

Statistical analysis The analysis of data was carried out by PRISM 3.0 using one-way ANOVA (nonparametric) and Dunnett's multiple comparison test. $P < 0.05$ was considered as low significant (*) while $p < 0.01$ – 0.001 were considered as highly significant (**/**).

Results

In vitro activity on adult *B. malayi*

The crude aqueous ethanolic extract of fruit of *Xylocarpus* was found to be effective in killing adult *B. malayi* and microfilaria at 125 and 62.5 $\mu\text{g/ml}$ (LC100). The IC50 values were 15.46 and 13.17 $\mu\text{g/ml}$, respectively, which were based on inhibition in both motility and MTT reduction in case of LC100 and only motility in case of IC50 (since MTT reduction may not always correlate with the motility inhibition) mf on testing at various concentrations (500–7.8 $\mu\text{g/ml}$). The standard drug ivermectin killed adult worm at 7.8 $\mu\text{g/ml}$ (IC50=1.61 $\mu\text{g/ml}$) and mf at 125 $\mu\text{g/ml}$ (IC50=3.62 $\mu\text{g/ml}$) concentration while DEC was inactive in vitro against both. Four fractions isolated from the crude extract viz. ethyl acetate-soluble fraction, *n*-butanol-soluble fraction, water-soluble fraction and water-insoluble fraction, exhibited in vitro antifilarial activity on parasites yielding variable IC50 values (adult worm, 8.50, 28.42, 20.6 and 19.08 $\mu\text{g/ml}$, respectively) and mf (6.9, 12.24, 29.68 and 28.72 $\mu\text{g/ml}$, respectively; Table 1). Of these, ethyl acetate-soluble fraction was found to be the

most active fraction and eight pure compounds were isolated from this fraction. However, six compounds were inactive up to 500 $\mu\text{g/ml}$ (the highest concentration tried) while remaining two molecules gedunin and photogedunin revealed promising in vitro activity on both adult parasite and microfilaria. These two compounds caused complete immobility of adult worm and mf in vitro up to as low as 0.98 and 3.9 $\mu\text{g/ml}$, respectively, and resulted in to >50% inhibition in reduction of MTT by adult parasite (Table 2). The IC50 values of the two compounds gedunin and photogedunin were quite close (adult, 0.24 and 0.21 $\mu\text{g/ml}$; mf, 2.03 and 2.23 $\mu\text{g/ml}$, respectively). The CC50 values of crude extract, fractions and pure molecules were above 200 $\mu\text{g/ml}$ and therefore the selectivity indices (SI) were found safe (Table 1) permitting further in vivo evaluation.

In vivo microfilaricidal activity

Microfilarial density in the tail blood of *Mastomys* after 5 days' treatment with the crude extract showed a gradual and continuous rise; however, the microfilarial densities remained below than that of control at any time point. As expected, treatment with the standard microfilaricide DEC led to a significant reduction in microfilaraemia on day 8/15 and the count rose thereafter although remained lower than pretreatment level up to day 45. In general, the microfilarial density of fraction-treated groups was lower than the untreated group. In case of *Mastomys* treated with ethyl acetate fraction, the progressive rise in microfilaraemia was comparatively lower than other fractions or the crude extract-treated groups. Animals administered with water-soluble fraction initially revealed suppressed mf density (Table 3).

Macrofilaricidal activity

Mastomys model In vivo antifilarial efficacy of the crude ethanolic extract and the four chromatographic fractions was evaluated on adult parasites of *B. malayi* in *Mastomys* at 250 mg/kg p.o. for five consecutive days. The crude aqueous ethanolic extract exerted 52.87 \pm 11.5% adulticidal activity and 62.70 \pm 7.0% embryostatic activity while its ethyl acetate-soluble fraction showed much inferior action on adult parasites (27.7 \pm 17.5% reduction). All the four fractions contained moderate degree of embryostatic action ranging between 37.3% and 40.4%. DEC which is principally microfilaricidal exhibited 50.2 \pm 6.7% adulticidal activity with sterilization of 37.82 \pm 9.5% of the recovered live females (Table 4).

Jird model Since major concentration of pure compounds was present in the ethyl acetate-soluble fraction, eight

Table 1 In vitro activity of the crude extract, fractions and pure compounds of the fruit portion of *X. granatum* on *B. malayi*

Test samples	Adult worm ($\mu\text{g/ml}$)			Microfilaria ($\mu\text{g/ml}$)	
	LC100; IC50	CC50	SI	LC100; IC50	SI
Crude aqueous ethanolic extract	125.0; 15.46	>300	>19.40	62.5; 13.17	>22.78
Ethyl acetate fraction	62.5; 8.50	>300	>35.29	31.25; 6.90	>43.47
<i>n</i> -Butanol fraction	125.0; 28.42	>300	>10.56	62.5; 12.24	>24.51
Water-soluble fraction	250.0; 20.60	>300	>14.56	125.0; 29.68	>10.11
Water-insoluble fraction	500.0; 19.08	>300	>15.72	250.0; 28.72	>10.45
Gedunin (pure compound)	0.98; 0.24	212.5	889.9	3.9; 2.03	435.58
Photogedunin (pure compound)	0.98; 0.21	62.3	1231.4	3.9; 2.23	241.09
Ivermectin (in vitro standard)	7.8; 1.61	52.84	20.36	125.0; 3.62	14.59
DEC	Inactive	>300	–	Inactive	–

compounds were isolated from this fraction. Only two (gedunin and photogedunin) of the eight pure compounds exhibited promising in vitro adulticidal activity, therefore were followed up in adult *B. malayi* transplanted jird in model at 100 mg/kg, s.c. for 5 days. Gedunin caused killing of 80.0% of the transplanted adult worms while photogedunin brought about 70.0% adult worm mortality; however, no embryostatic activity in any of the two pure compounds was noticed in jirds. The number and the degree of motility of mf on autopsy at the end of observation period (day 50) in case of both the pure compounds was tremendously decreased when compared with that of controls (60–70%) demonstrating adverse effect of pure molecules on microfilarial stages.

DEC administered by the same route (s.c.) at 100 mg/kg for 5 days revealed mere 30% adulticidal activity without female worm sterilization (Table 4).

Discussion

Helminth parasites including filarial nematodes represent major cause of human misery as ascarids, hookworm and filarial infections are ubiquitous in developing nations causing severe disfiguration and disability. The nature proves itself as the major reservoir of products medicinally used against many dreadful diseases. The extracts of plant parts serve as a major resource for development of leading drugs against troublesome diseases. A preliminary report by Zaridah et al. (2001) on in vitro antifilarial evaluation of crude extract from seed, leaves and husk of *X. granatum* at very high concentrations shows an adverse effect of seed on the motility of adult *B. malayi*. However, they neither tested the fruit part nor purified the crude extract or performed any in vivo studies. The present study involves fruit portion of this plant collected from Andaman coastal region and the crude extract was evaluated for its in vitro as well as in vivo

activity against *B. malayi* parasite and mf. Based on the results obtained from in vitro experiments, bioactivity-guided fractionation was carried out in order to locate the antifilarial activity in the active fraction and thereafter in pure compound/s. The in vivo evaluation of the crude extract and various fractions was then carried out. The pure molecules isolated were first tested in vitro and the active ones were followed for in vivo evaluation. Of the eight, only two pure compounds gedunin and photogedunin were found to possess significant adulticidal and microfilaricidal activity in vitro and their IC₅₀ was quite low (0.239, 0.213 $\mu\text{g/ml}$ for adults and 2.03; 2.23 $\mu\text{g/ml}$ for mf, respectively). Both these molecules possessed selectivity index of >800 thus pursued further for in vivo evaluation. The crude aqueous ethanolic extract were administered orally in *M. coucha* at 5×250 mg/kg which exerted 52.9% adulticidal and 62.7% embryostatic action when the animals were euthanized on day 90 post-initiation of 5-days treatment. The microfilarial densities were also marginally reduced as compared to those of control *Mastomys*. Nevertheless, almost all the fractions except *n*-butanol soluble revealed some effect on microfilaraemia especially after treatment as compared to the crude extract, *n*-butanol data was almost comparable to the crude test sample. Since the crude extract revealed moderate in vivo antifilarial action in *Mastomys*, the various fractions were tried at the same dose without taking any risk of losing antifilarial activity, if at all present, in these fractions. Surprisingly, the antifilarial action got distributed in the fractions resulting in to adulticidal efficacy much inferior to the crude extract. Amongst all, ethyl acetate-soluble fraction appeared to be the most active one and therefore repeat tested. The gedunin and photogedunin are known compounds; however, till date, no report is available on their antifilarial activity whether in vitro or in vivo. Gedunin is known to inhibit the growth of CaCo-2 colon cancer cell line (Uddin et al. 2007) and protects the gastric mucosa of peptic ulcer in rats by exhibiting significant

Table 2 Concentration-dependent in vitro activity of *X. granatum* crude extract, fractions and pure compounds on *B. malayi* adult and microfilaria

Test samples	Concentration (ug/ml)	Adult		Microfilariae Motility score ^a
		Motility score ^a	Percentage of inhibition in MTT reduction over control	
Crude aqueous ethanolic extract	125.0	D	50	D
	62.5	1+	46.3	D
	31.25	2+	23.7	1+
Ethyl acetate fraction	62.5	D	68.8	D
	31.25	1+	45.0	D
	15.6	1+	40.2	1+
	7.8	2+	35.8	2+
<i>n</i> -Butanol fraction	125.0	D	62.5	D
	62.5	1+	27.5	D
	31.25	2+	18.8	1+
	15.6	1+	40.2	1+
Water-soluble fraction	250.0	D	59.4	D
	125.0	1+	28.8	D
	62.5	1+	23.1	1+
	31.25	2+	36.4	2+
Water-insoluble fraction	500.0	D	68.6	D
	250.0	1+	50.0	D
	125.0	1+	67.2	1+
	62.5	2+	35.2	2+
Gedunin (pure compound)	3.9	D	96.4	D
	1.9	D	95.7	2+
	0.98	D	84.2	3+
	0.49	1+	20.8	3+
Photogedunin (pure compound)	3.9	D	96.2	D
	1.9	D	92.5	2+
	0.98	D	90.3	3+
	0.49	1+	39.8	3+
Ivermectin (standard drug)	7.8	D	50.0	3+
	3.9	1+	28.6	3+
	1.9	1+	20.8	3+
	0.98	2+	18.8	3+
Control 1 (vehicle)	–	4+	–	4+
Control 2 (vehicle)	–	4+	–	4+
Control 3 (vehicle)	–	4+	–	4+

^a Reduction in motility of parasite: 4+, 0%; 3+, 1–49%; 2+, 50–74%; 1+, 75–99%; D, 100%

anti-secretory effects (Lakshmi et al. 2010). At 5×100 mg/kg, subcutaneous adult *B. malayi* i.p. transplanted in the jird model, these compounds exhibited very promising activity on adult parasite causing 80.0% mortality of adult worms by gedunin and 70.0% by photogedunin. There was no embryostatic activity when animals were euthanized on day 50. The microfilariae were not checked on day 8 of start of treatment unlike *Mastomys* to avoid any injury to implanted worm during frequent withdrawal of peritoneal fluid. Nevertheless, peritoneal fluid was examined on day 50 during animal autopsy which revealed 60–70% decrease in microfilarial density in the fluid as also similar degree of

reduced motility. DEC was used as standard filaricidal drug in vivo as it is a strong microfilaricidal in *B. malayi/Mastomys* model, imparting partial adulticidal and embryostatic action (Singh et al. 1997) which Ivermectin possesses in vitro microfilaricidal (personal observation) and macrofilaricidal (Lakshmi et al. 2009) action on *B. malayi*, and therefore was selected as a standard drug in in vitro screen. It is well known that DEC is ineffective in vitro (Misra-Bhattacharya et al. 2004); however, it was included as a standard drug along with ivermectin in vitro for comparison. For in vivo evaluation, two animal models were included in the present study (1) subcutaneous *B.*

Table 3 Effect of *X. granatum* crude extract and fractions on microfilaraemia in *B. malayi* infected *M. coucha*

Test samples	Dose (mg/kg), p.o.×5 days	No. of animals used	% Change (mean±SE) in mf per 10 µl tail blood post initiation of treatment (days)						
			8	15	30	45	60	75	90
Crude aqueous ethanolic extract	250	3×2	+28.0 ±40.1	+47.1 ±71.0	+127.6 ±84.5	+139.3 ±97.3	+365.5 ±222.2	+483.7 ±282.1	+432.2 ±265.2
Ethyl acetate fraction	250	3×2	+0.7 ±5.7	+15.9 ±21.7	+14.0 ±15.5	+24.9 ±7.3	+34.8 ±9.1	+94.5 ±36.9	+119.8* ±43.0
<i>n</i> -Butanol fraction	250	3×1	+41.2 ±36.1	+109.8 ±28.0	+108.6 ±50.9	+143.1 ±95.6	+182.8 ±142.4	+282.8 ±1.0	+323.4 ±25.2
Water-soluble fraction	250	3×1	-1.9 ±9.3	-0.5 ±4.5	+16.7 ±29.9	+147.9 ±55.2	+207.1 ±44.3	+195.6 ±35.3	+205.1* ±30.4
Water-insoluble fraction	250	3×1	+5.2 ±17.1	+10.8 ±29.1	+29.7 ±45.6	+161.4 ±126.6	+276.0 ±138.2	+235.4 ±127.3	+200.1* ±109.3
DEC (in vivo standard)	50	5×1	-79.0* ±5.5	-82.7* ±82.6	-74.7* ±7.4	-15.3* ±37.3	+27.9 ±46.9	+210.9 ±138.0	+116.3 ±71.7
Control	–	5×2	+37.8 ±12.7	+87.5 ±19.9	+205.5 ±50.8	+304.8 ±60.0	+460.0 ±98.5	+717.6 ±181.2	+1061.0 ±378.1

**P*<0.05 low significant

malayi L3 induced infection in *M. coucha* and (2) adult *B. malayi* i.p. transplanted in the jird model. The former route of infection is natural and develops into a chronic infection akin to humans while infection in jird by i.p. implantation of adult parasites is conveniently used as a primary in vivo screen because of being less time

consuming and giving results in much shorter time with accurate assessment of adulticidal effects since a known number of adult worms are implanted. The high selectivity index and very low IC50 makes these two compounds of natural product origin, very interesting and promising as potential antifilarial agents against human lymphatic

Table 4 Macrofilaricidal (adulticidal) activity of crude extract, fractions and pure compounds of *X. granatum* fruit against *B. malayi* in *Mastomys*/transplanted jird model by subcutaneous (s.c.) and oral (p.o.) route

Test samples	Dose (mg/kg,×5 days)	No. of animals	% change in worm recovery and sterilization over control			
			Male	Female	Total	% Female worms sterilized
<i>M. coucha</i> —subcutaneous L3-induced model						
Crude aqueous ethanolic extract	250 p.o.	3×2	-56.25±16.1	-51.22±11.3	-52.87±11.5**	+62.70±7.0**
Ethyl acetate fraction	250 p.o.	3×2	-20.8±4.2	-34.2±23.9	-27.7±17.5	+37.7±16.9
<i>n</i> -Butanol fraction	250 p.o.	3×1	+41.7±41.0	-44.8±11.8	-15.6±21.2	+37.3±19.1
water-soluble fraction	250 p.o.	3×1	+37.5±62.9	-19.3±28.1	-0.1±38.8	+40.4±27.7
water-insoluble fraction	250 p.o.	3×1	-4.2±11.02	-23.53±25.5	-1.8±21.6	+38.23±10.2
DEC	50 p.o.	5×1	-60.0±4.7	-45.2±8.5	-50.2±6.7**	+37.82±9.5**
Untreated Control	–	5×2	8.0	15.7	23.7	15.1
Adult <i>B. malayi</i> i.p. transplanted in jird model						
Photogedunin	100 s.c.	3×2	-100±0.0	-53.9±15.3	-70.0±10.0**	nil
Gedunin	100 s.c.	3×2	-85.7±14.3	-76.9±7.7	-80.0±10.0**	nil
DEC	100 s.c.	3×2	-33.4±9.5	-29.2±13.5	-30.0±10.0*	nil
Untreated Control	–	3×2	3.5	6.5	10	3.5

Percent reduction in worm recovery has been assessed by comparing the mean values of treated group with that of respective untreated control group. Statistical analysis was done by comparing each treated group vs. respective control using one-way ANOVA (nonparametric) and Dunnett's multiple comparison test.

Statistical significance, **P*<0.05 is low significance; ***P*<0.01 is highly significant

filariasis. A number of protolimonoids, limonoids (Cui et al. 2008; Yin et al. 2007), lignins, tannins (Shinoda et al. 1985), alkaloids (Chou et al. 1977) and sterols (Hogg and Gillian 1984) have also been reported from *X. granatum*. Essential oils β -selinene and γ -selinene have also been isolated from the fruits and leaves (Du et al. 2007). Methylflindersine and xylococcins Q-V (34–39) isolated from this plant has anti-feedant (Wu et al. 2005), insect repellent, antimicrobial, anti-yeast and antifungal (Chou et al. 1977; Bandaranayake 2002; Du et al. 2009) activities. Thus, broad spectrum of biological activities present in this plant makes it an interesting mangrove to be exploited further.

It may be surmised that *X. granatum* possessed promising activity against *B. malayi* parasite in the two experimental models. This is the first ever report on the adulticidal antifilarial activity of gedunin and photogedunin isolated from this mangrove. The activity of the two compounds in subcutaneous L3 induced in *Mastomys* model and a dose-dependent response is underway. The above findings are very encouraging and advocate further synthesis of the chemical analogues of the two compounds and their antifilarial evaluation.

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