*Parasitology Research 2011, 109(5), 1351-1360*

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

**[a]Sweta Misra, [a]Meenakshi Verma, [b]Sunil Kumar Mishra, [b]Shishir Srivastava, [b]Vijai Lakshmi and [a]Shailja Misra-Bhattacharya[*]**

Divisions of Parasitology[a] and Medicinal and Process Chemistry[b], Central Drug Research Institute, M. G. Marg, Lucknow (U.P.) 226001, India

[*]Corresponding author: Dr. Shailja Misra-Bhattacharya
FAX: +91-522-2623405/2623938/2629504, phone: +91-522-2612411-18 PABX 4221/4224, E mail: shailja_cdri@rediffmail.com/shailjacdri@yahoo.com

## 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 *Xylocarpus 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* i.p. transplanted jird model (*Meriones unguiculatus*) and *Mastomys coucha s*ubcutaneously 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 5x250 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, CC50=212.5, SI=889.1) and Photogedunin (IC50=0.213, CC50=262.3, SI=1231.4) at 5x100 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 *Xylocarpus 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.

**Key words:** *Brugia malayi*, mastomys, jird, Gedunin, Photogedunin, *Xylocarpus granatum,* antifilarial, macrofilaricidal.

## Introduction

Human lymphatic filariasis a vector-borne disease mainly caused by *Wuchereria bancrofti*, *Brugia malayi* and *Brugia timori* continues to cripple population in the tropical and subtropical countries. *Wuchereria 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 Diethylcarbamazine 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.

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 grape fruit size, hard and heavy, leading to the common name 'cannon ball tree' (Mullholand and Taylor 1992). 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, *Brugia 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 (1.0Kg) was extracted with 50% aqueous ethanol 5 X 5.0 L. and the combined extracts were filtered, concentrated under reduced pressure below $50^0$C to a minimum volume of 1.0 lit. It was further dried in hot air vacuum oven at $45^0$C to brown powder (yield 15%).The brown powder was further fractionated into chloroform soluble (yield 8.5% of the 50% aq. 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 $C_{18}$ R.P columns using acetonitrile-water 55:45, v/v, flowrate-1.0 ml/min using UV detector (λ 230 nm) yielded compounds namely Gedunin (36%) (Taylor 1974), Photo-gedunin (2%) (Lakshmi et al. 2010) as shown in Fig.1. All these were characterized using IR, NMR, mass, derivetization 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.

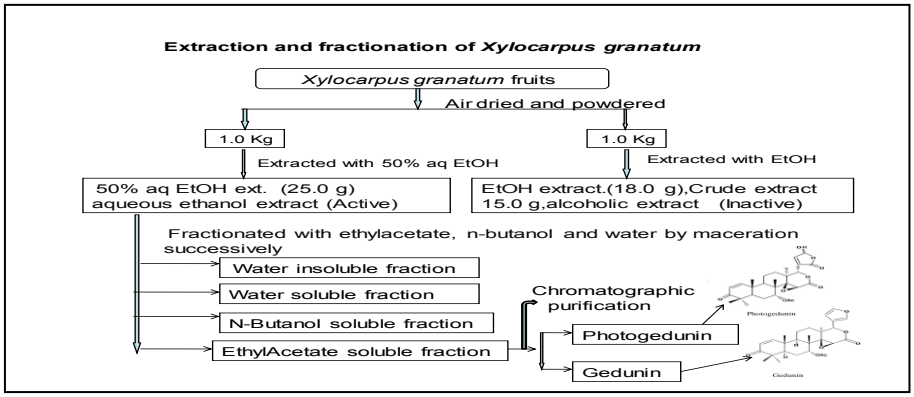


**Fig-1** Extraction and Fractionation of X. granatum

## 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* while jird received 150 L3 intraperitoneally. 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 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 two-fold dilutions of the stock starting from 500 µg/ml to the lowest concentration of 3.9 µg/ml. The pure compounds were tested at various two fold dilutions starting from 31.25 µg/ml to 0.12 µg/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 1000 µl media with one female parasite /well (NUNC). RPMI 1640 medium containing antibiotics (penicillin 100 units/ml, streptomycin sulphate 100 µg/ ml,and neomycin mixture; Sigma, USA) and fortified with10% fetal bovine serum was used. The worms were exposed to test sample for 48 hours at $37^0$C in $CO_2$ 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 two-fold dilutions (125 to 0.12 µg/ml) of stock solution of compounds were added. The incubation conditions were same as 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 µg/ml and IC50 mf 3.62 µg/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 ≥50% inhibition in MTT reduction by treated parasite over untreated control (Mukherjee et al. 1998).

***Evaluation of IC50*** For assessing IC50, further two fold dilutions of each test material was tested starting from LC100 value up to 0.3 µg/ml. IC50 conc. is determined by Excel based line graphic template after plotting conc. values of each sample against percent inhibition in motility of parasite on x- and y axis. 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 values. Vero cells (monkey kidney cell line) in culture flask were trypsinized and counted using Neubauer chamber. 6.5 ml of cell suspension / plate ($10^5$ cells /ml) is required for assessing CC50 in a 96 well plate format. 100 µl/ well of Minimum essential medium (MEM) was added to each third column of 96 well culture plate without vero cells to serve as negative control. 100 µl of the above cell suspension is plated in to each well except $3^{th}$, $6^{th}$, $9^{th}$ and $12^{th}$ column and incubated overnight at $37^0$C in $CO_2$ incubator. Medium was removed from all the wells and replaced by 100 µl of fresh medium. 150 µl medium containing 300 µg/ml test sample (highest concentration) was added to row-H in triplicates (i.e. compound 1 from column 1 to 3, compound 2 from column 4 to 6, compound 3 from column 7 to 9 and compound 4 from column 10 to 12). Serial dilutions (3:1) were prepared with a multichannel pipette by transferring 50 µl from row-H to row-G and mixing it and re-transferring 50 µl 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 $37^0$C in 5% $CO_2$ in air. After 72 h of drug exposure, 10 µl /well of viability marker dye Resazurin or Alamar blue (stock solution 12.5 mg / 100 ml PBS) was added and plate is 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 (SI)*** The SI values of the *in vitro* active extracts was determined (SI=CC50/IC50) and SI ≥ 10 was considered as promising and safe for pushing the sample 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 jird:* 6-8 week old male jird was intraperitoneally transplanted with 10 females and 5 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 L3 infected Mastomys coucha:* Mastomys were infected by subcutaneous inoculation of ~100 infective larvae each recovered from *Aedes aegypti* fed on donor mastomys 8/9 days back (Singh et al. 1997). Animals were monitored for microfilaraemia in 10 µl tail blood between 12.00 and 13.00 h 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 5 consecutive days at 250 mg/ kg body weight, while the pure compounds were given at 100 mg/kg x 5 consecutive days by subcutaneous (s.c.) route in i.p. adult transplanted 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 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 µl 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. 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 percent sterile females was 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 3 mastomys (experiment not repeated due to inferior activity) while for standard drug DEC only 5 mastomys were used in a single experiment. Control mastomys group receiving vehicle consisted of 5 animals each in duplicate experiments.

Regarding jirds, 3 i.p. transplanted 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 ANNOVA (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 µg/ml (LC100). The IC50 values were 15.46 and 13.17 µg/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 µg/ml). The standard drug ivermectin killed adult worm at 7.8 µg/ml (IC50=1.61 µg/ml) and mf at 125 µg/ml (IC50=3.62 µg/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 µg/ml respectively) and mf (6.9, 12.24, 29.68 and 28.72 respectively) (Table 1a). 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 to500 µg/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 µg/ml respectively and resulted in to >50 % inhibition in reduction of MTT by adult parasite (Table1b). The IC50 values of the two compounds Gedunin and Photogedunin were quite close (adult- 0.24 µg/ml and 0.21 µg/ml; mf- 2.03 and 2.23 µg/ml respectively). The CC50 values of crude extract, fractions and pure molecules were

above 200 µg/ml and therefore the selectivity indices (SI) were found safe (Table 1a) permitting further *in vivo* evaluation.

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

| Test samples | Adult worm (µg/ml) | | | Microfilaria (µg/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 |
| **n-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 | - |

***In vivo microfilaricidal (MIF) 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 rise 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 2).

| Test samples | Conc. ug/ml | Adult | | Microfilariae |
|---|---|---|---|---|
| | | Motility score* | % inhibition in MTT reduction over control | Motility score * |
| **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+ |
| **n-butanol fraction** | 125.0 | D | 62.5 | D |
| | 62.5 | 1+ | 27.5 | D |
| | 31.25 | 2+ | 18.8 | 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+ |

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

* reduction in motility of parasite: 4+, 0%; 3+, 1-49%; 2+, 50-74%; 1+, 75-99%; D, 100%

***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. x 5 consecutive days. The crude aqueous ethanolic extract exerted 52.87±11.5% adulticidal activity and 62.70±7.0% embryostatic activity while its ethyl acetate soluble fraction showed much inferior action on adult parasites (27.7±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±6.7% adulticidal activity with sterilization of 37.82±9.5% of the recovered live females (Table 3).

***Jird model*:** Since major concentration of pure compounds was present in the ethyl acetate soluble fraction, eight 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 model at 100 mg/kg, s.c. x 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 3).

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

| Test samples | Dose mg/kg, p.o.x5day | No. of animal used | % change (mean±SE ) in Mf/10 µl tail blood post initiation of treatment - on days | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | 8 | 15 | 30 | 45 | 60 | 75 | 90 |
| **Crude aqueous ethanolic extract** | **250** | **3x2** | +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** | **3x2** | +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 |
| **n-butanol fraction** | **250** | **3x1** | +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** | **3x1** | -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** | **3x1** | +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** | **5x1** | -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** | **-** | **5x2** | +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

**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, x 5 days) | No. of animals | % change in worm recovery and sterilization over control | | | |
|---|---|---|---|---|---|---|
| | | | male | female | total | % female worms sterilized |
| ***Mastomys coucha* - subcutaneous L3 induced model** | | | | | | |
| **Crude aqueous ethanolic extract** | 250 p.o. | 3x2 | -56.25±16.1 | -51.22±11.3 | -52.87±11.5** | +62.70±7.0** |
| **Ethyl acetate fraction** | 250 p.o. | 3x2 | -20.8±4.2 | -34.2±23.9 | -27.7±17.5 | +37.7±16.9 |
| **n-butanol fraction** | 250 p.o. | 3x1 | +41.7±41.0 | -44.8±11.8 | -15.6±21.2 | +37.3±19.1 |
| **water soluble fraction** | 250 p.o. | 3x1 | +37.5±62.9 | -19.3±28.1 | -0.1±38.8 | +40.4±27.7 |
| **water insoluble fraction** | 250 p.o. | 3x1 | -4.2±11.02 | -23.53±25.5 | -1.8±21.6 | +38.23±10.2 |
| **DEC** | 50 p.o. | 5x1 | -60.0±4.7 | -45.2±8.5 | -50.2±6.7** | +37.82±9.5** |
| **Untreated Control** | - | 5x2 | 8.0 | 15.7 | 23.7 | 15.1 |
| **Adult *B. malayi* I.P. transplanted jird model** | | | | | | |
| **Photogedunin** | 100 s.c. | 3x2 | -100±0.0 | -53.9±15.3 | -70.0 ±10.0** | nil |
| **Gedunin** | 100 s.c. | 3x2 | -85.7±14.3 | -76.9±7.7 | -80.0±10.0** | nil |
| **DEC** | 100 s.c. | 3x2 | -33.4±9.5 | -29.2±13.5 | -30.0±10.0* | nil |
| **Untreated Control** | - | 3x2 | 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 Annova (nonparametric) and Dunnett's multiple comparison test.
Statistical significance - * P <0.05 is low significance; ** P <0.01 is highly significant.

## 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 for adults and 2.03;2.23 for mf respectively). Both these molecules possessed selectivity index of >800 thus pursued further for *in vivo* evaluation. The crude aqueous ethanolic extract when administered orally in *Mastomys coucha* at 5x250 mg/kg exerted 52.9% adulticidal and 62.7% embryostatic action in addition to marginally reduced microfilarial densities. 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 anti-secretory effects (Lakshmi et al. 2010). At 5x100 mg/kg, subcutaneously in i.p. adult *B. malayi* transplanted 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 strong microfilaricidal in *B. malayi* /mastomys model imparting partial adulticidal and embryostatic action. Ivermectin possesses micro- and macrofilaricidal action on *B. malayi in vitro* and therefore was selected as a standard drug in *in vitro* screen. It is well known that DEC is ineffective *in vitro*, however, it was included as standard drug along with ivermectin *in vitro* for comparison. For *in vivo* evaluation, two animal models were included in the present study i) subcutaneous *B. malayi* L3 induced infection in *Mastomys coucha* and ii) i.p. adult *B. malayi* transplanted 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 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 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 xyloccensins Q-V (34–39) isolated from this plant has anti-feedant (Wu et al. 2005), insect repellent, antimicrobial, anti-yeast and antifungal (Chou 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 *Xylocarpus 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 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.

**Acknowledgements**

The study was supported by Department of Ocean Development, New Delhi, India. We thank Mr. A. K. Roy and Mr. R. N. Lal for providing technical assistance. The financial assistance in form of fellowship to S.M. by Council of Scientific and Industrial Research, New Delhi, India and to M.V. by World Health Organization, Geneva under CDRI-WHO collaborative project (A70112) is gratefully acknowledged. **CSIR-CDRI Communication No. 8034**

**References**


- Alam MA, Sarder M, Awal MA, Sikder MMH, Daulla KA (2006) Antibacterial activity of the crude ethanolic extract of *Xylocarpus granatum* stem barks. Bangl J Vet Med 4 (1): 69–72
- Alvi KA, Crews P, Aalbersberg B, Prasad R (1991) Limonoids from the Fijian medicinal plant dabi (*Xylocarpus*). Tetrahedron 47: 8943–8948
- Bandaranayake WM (2002) Bioactivities, bioactive compounds and chemical constituents of mangrove plants. [Review article]. Wetlands Ecology and Management 10: 421-452.
- Chopra RN, Nayar SL, Chopra IC (1956) Glossary of Indian Medicinal Plants, CSIR, New Delhi
- Chou FY, Hostettmann K, Kubo I, Nakanishi K, Taniguchi M (1977) Isolation of an insect antifeedant N-methylflindersine and several benz[c]phenanthridine alkaloids from East African plants; a comment on chelerythrine. Heterocycles 7 (2): 969–977
- Cui J, Ouyang J, Dengb Z, Lin W (2008) Structure elucidation of an unprecedented alkaloid and a new limonoid from *Xylocarpus granatum*. Magn Reson Chem. 46: 894–897
- Du SJ, Qin ZH, Wang MA, Zhu W, Han CR, Bi HP (2007) GC-MS analysis of the essential oils from *Xylocarpus granatum*. J Hainan Normal Univ 20(4): 247–250
- Du S, Wang M, Zhu W, Qin Z (2009) A new fungicidal lactone from *Xylocarpus granatum* (Meliaceae). Natural Product Res 23: 1316–1321
- Dutta A, Sukul NC (1987) Antifilarial effect of *Zingiber officinale* on *Dirofilaria immitis*. J Helminthol 61(3): 268-70
- Hogg RW, Gillian FT (1984) Fatty acids, sterols and hydrocarbons in the leaves from eleven species of mangrove. Phytochemistry 23: 93-97
- Lakshmi V, Gupta P (2008) An overview of the genus *Xylocarpus*. Nat Prod Res 22 (14): 1197-224
- Lakshmi V, Srivastava S, Mishra SK, Misra S, Verma M, Misra-Bhattacharya S (2009) *In vitro* and *in vivo* antifilarial potential of marine sponge, *Haliclona exigua* (Kirkpatrick), against human lymphatic filarial parasite *Brugia malayi*. Parasitol Res 105: 1295-1301
- Lakshmi V, Singh N, Shrivastva S, Mishra SK, Dhamani P, Mishra V, Palit G (2010) Gedunin and photogedunin of *Xylocarpus granatum* show significant anti-secretory effects and protect the gastric mucosa of peptic ulcer in rats. Phytomedicine 17: 569-574
- McCall JW, Malove JB, Hyong-Sun A, Thompson PE (1973) Mongolian jirds (*Meriones unguiculatus*) infected with *Brugia malayi* by the intraperitoneal route. A rich source of developing larvae, adult filariae and mf. J Parasitol 59 (3): 436
- Misra S, Chatterjee RK, Sen AB (1984) The response of *Litomosoides carinii* to antifilarial agents in cotton rat *Sigmodon hispidus* and multimammate rat (*Mastomys natalensis*). Indian J Med Res 7: 749-752
- Misra-Bhattacharya S, Katiyar D, Bajpai P, Tripathi RP, Saxena JK (2004) 4-Methyl-7-(tetradecanoyl)-2H-1-benzopyran-2-one a novel DNA topoisomerase II inhibitor with adulticidal and embryostatic activity against sub-periodic *Brugia malayi*. Parasitol Res 92: 177-182
- Mukherjee M, Misra S, Chatterjee RK (1998) Development of *in vitro* screening system for assessment of antifilarial activity of compounds. Acta Trop 70 (3): 251-255
- Omar S, Godard K, Ingham A (2003) Antimalarial activities of gedunin and 7-methoxygedunin and synergistic activity with dillapiol. Ann Appl Biol 143: 135–141
- Ottesen EA (2000) The global programme to eliminate lymphatic filariasis. Trop Med Int Hlth 5: 591–594
- Rouf R, Uddina SJ, Shilpi JA, Alamgir M (2007) Assessment of antidiarrhoeal activity of the methanol extract of *Xylocarpus granatum* bark in mice model. J Ethanopharmacol 109: 539-542
- Shinoda Y, Ogisu M, Iwata S, Tajima T (1985) Chemical composition of mangroves. II. Gifu Daigaku Nogakubu Kenkyu Hokoku 50: 155-165
- Singh VK, Ram ER (1988) Filaria and its herbal cure. New Botanist 15: 201-205

- Singh U, Misra S, Murthy PK, Katiyar JC, Agarwal A, Sircar AR (1997) Immunoreactive molecules of *Brugia malayi* and their diagnostic potential.Serodiag Immunother Infect Dis 8: 207- 212

- Taylor DAH (1974) $^{13}C$ Nuclear Magnetic Resonance spectra of some limonoids. Part-1. The structure of Procerin, an extractive from *Carapa procera*. J Chem Soc Pt 1: 437-441
- Titanji VPK, Evehe MS, Ayafor JF, Kimbu SF (1990) Novel *Onchocerca volvulus* filaricides from *Carapa procera*, *Polyalthia suaveolens* and *Pachypodanthium staudtii*. Acta Leidensia 59: 377-382
- Uddin SJ, Nahar L, Shilpi JA, Shoeb M, Borkowski T, Gibbons S, Middleton M, Byres M, Sarker S D (2007) Gedunin a Limonoid from *Xylocarpus granatum*, Inhibits the Growth of CaCo-2 Colon Cancer Cell Line *In Vitro.* Phytother Res 21: 757-761
- Wu J, Xiao Q, Zhang S, Li X, Xiao Z, Ding H, Lia Q (2005) Xyloccensins Q-V, six new 8,9,30-phragmalin ortho ester antifeedants from the Chinese mangrove *Xylocarpus granatum*. Tetrahedron 61: 8382–8389
- Yin S, Wang X-N, Fan C-Q, Lin L-P, Ding J, Yue J-M (2007) Limonoids from the seeds of the marine mangrove *Xylocarpus granatum* . J Nat Prod 70 (4): 682-685
- Yusuf M, Chowdhury JU, Wahab MA, Begum J (1994) Medicinal Plants of Bangladesh, BCSIR Laboratories, Chittagong, Bangladesh p. 263.
- Zaridah MZ, Idid SZ, Wan-Omar A, Khozirah S (2001) *In vitro* antifilarial effects of three plant species against adult worms of sub periodic *Brugia malayi*. J Ethnopharmacol 78: 79–84