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ARTICLE *in* PARASITOLOGY RESEARCH · AUGUST 2009

Impact Factor: 2.1 · DOI: 10.1007/s00436-009-1555-1 · Source: PubMed

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In-vitro and *in-vivo* antifilarial potential of marine sponge, *Haliclona exigua* (Kirkpatrick) against human lymphatic filarial parasite *Brugia malayi*

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Abstract:

The present study reports on the antifilarial activity of a marine sponge *Haliclona exigua* (phylum-Porifera). The crude methanol extract and n-butanol soluble fraction killed adult *B. malayi* at 31.25µg/ml concentration (both in motility and MTT assay) while the chloroform fraction was lethal at a lower conc. of 15.6µg/ml. The activity could be located in a single molecule araguspongin C which brought about mortality of worm at 15.6µg/ml. *In vivo* evaluation of the crude extract (5x500mg/kg,orally) and the chloroform fraction (5x250mg/kg,orally) in *Brugia malayi* infected rodent host, *Mastomys coucha* did not show any significant microfilaricidal actions, however, microfilarial densities in both the treated groups were significantly much lower than those of untreated group in contrast to standard filaricide diethylcarbamazine (DEC) which exerted 79% microfilaricidal action on day 8 of treatment. Both these extracts also demonstrated adulticidal (macrofilaricidal) activity which was more pronounced in the chloroform fraction (50.2%). In addition, there was moderate adverse effect on the reproductive potential of female worms (crude extract: 46.5%; chloroform: 58.6 %). The findings suggest that the marine sponge *H. exigua* possesses adulticidal and embryostatic action against human lymphatic filarial parasite *B. ma*layi in experimental rodent model and this activity could be attributed to the presence of araguspongin C.

Keyword: *Brugia malayi ; Mastomys coucha ;* diethylcarbamazine

Introduction:

Lymphatic filariasis is a major vector-borne disease in the developing world. *Wuchereria bancrofti*, the predominant filarial parasite, affects more than 90% of lymphatic filarial patients, causing acute and chronic morbidity (World Health Organization 1994) in persons of all ages and both sexes. Diethylcarbamazine (DEC) and ivermectin remain the mainstay antifilarial drugs which are principally microfilaricidal with limited or no action on adult parasites. A drug which may either kill the adult parasite or adversely affect the reproductive potential of adult worms is therefore urgently needed. Marine resources offer an unprecedented opportunity for their pharmacological exploration and, hence, have received great attention during recent years for natural product chemistry. Marine microorganisms with immense genetic and biochemical diversity have recently been appreciated for being a rich source of novel chemical entities for the discovery of candidate drugs. Secondary metabolites produced in marine organisms could be the source of bioactive substances and useful in modeling compounds for drugs (Faulkner *2001*; Haefner 2003). Marine sponges are shown to exhibit antibacterial, insecticidal, antiviral,

antifungal, antifilarial, antileishmanial and antiplasmodial activities (Rao et al. 2003*;* Yan 2004*;* Lakshmi et al., 2004; Dube et al., 2007). We have earlier reported the antifilarial activity in various marine flora and fauna (Lakshmi et al, 2004 a,b). *Haliclona exigua* (Kirkpatrick), a sedentary marine sponge belonging to phylum Porifera, class Demospongia, order Haploscleridae, and family Halicloniidae was identified. This sponge sp. is found in sub-tidal region of Vallai Island, Setukarai, Gulf of Mannar, Ramnathpuram and Tamil Nadu coasts of India. Asymmetrical, amorphous, brownish yellow, colonies of irregularly rounded *Haliclona exigua* (4 - 10 cm) remain attached to sea bottom by means of masses of spicules and possess the corn type of canal system and are found attached on the dead coral stones in shallow water areas at the depth of 3–6 m. Some marine extracts have earlier been reported to possess anti-infective and anti-parasitic activity including antifilarial (Lakshmi et al., 2004a, b; Rao et al., 2003; 2004; Nakao et al, 2004; Yan 2004). *Haliclona* sp. has also been reported to act on *Aspergillus* strains (Bhosale et al. 1999). Few researchers tried to isolate the chemical constituent of the *Haliclona exigua* (Reddy et al. 1997; Venkateswarlu et al. 1994; Dube et al, 2007). The activity reported by the various workers in this sponge inhibited the rat brain nitric oxide synthase activity (Venkateswarlu et al. 1994).

The present communication deals with the evaluation of crude extract and various fractions as well as pure compound of the marine sponge, *Haliclona exigua* for antifilarial activity using the sub-periodic strain of human lymphatic filariid, *Brugia mala*yi both *in vitro* and *in vivo* in its experimental rodent host *Mastomys coucha*.

Materials and methods

2.1 Collection of material:

Haliclona exigua (Kirkpatrick) was identified by Dr. P. A. Thomas of the Central Marine and Fisheries Institute, Trivandrum, Kerala (India) and collected from Tamil Nadu coast of India in the month of August. Fresh sponges were filled in the steel containers containing methanol on Tamil Nadu coast of India and were transported to CDRI laboratory. Specimen sample (Voucher specimen No. 343) has been preserved in the Herbarium of Botany Division, Central Drug Research Institute, Lucknow, India.

In vitro efficacy

The crude extract and pure compound were prepared in minimum volume of DMSO and used at several two fold dilutions starting from 500 µg/ml with lowest concentration up to 15.6 ug/ml to determine the lethal concentration (LC100) and also to pick up the most active extract/fraction/pure compound. The parasites were isolated aseptically from gerbils infected intraperitoneally (McCall 1973) 90–150 days earlier with 200–250 L3 of *B. malayi*. The worms (one female worm/well) were incubated at 37^0C overnight in RPMI 1640 medium containing antibiotics (penicillin 100 units/ml, streptomycin sulphate 100 µg/ ml, and neomycin mixture; Sigma, USA), and various concentrations of each extract were added to the incubation mixture. After completion of the drug exposure time, the motility of worms was recorded microscopically 1 h after transfer to fresh drug-free medium. Parasites were processed for the 3-(4, 5 dimethylthiazol- 2-yl)-2, 5 diphenyl tetrazolium bromide (MTT) dye reduction assay as indicated previously (Mukherjee et al. 1998). Experiments were conducted in two replicates and the total loss of motility and percent inhibition in MTT reduction in treated parasites compared to untreated controls was assessed. The complete immobility of adult worm with ≥50% inhibition of MTT reduction by treated parasites compared to untreated controls was considered as adulticidal action (Mukherjee et al. 1998).

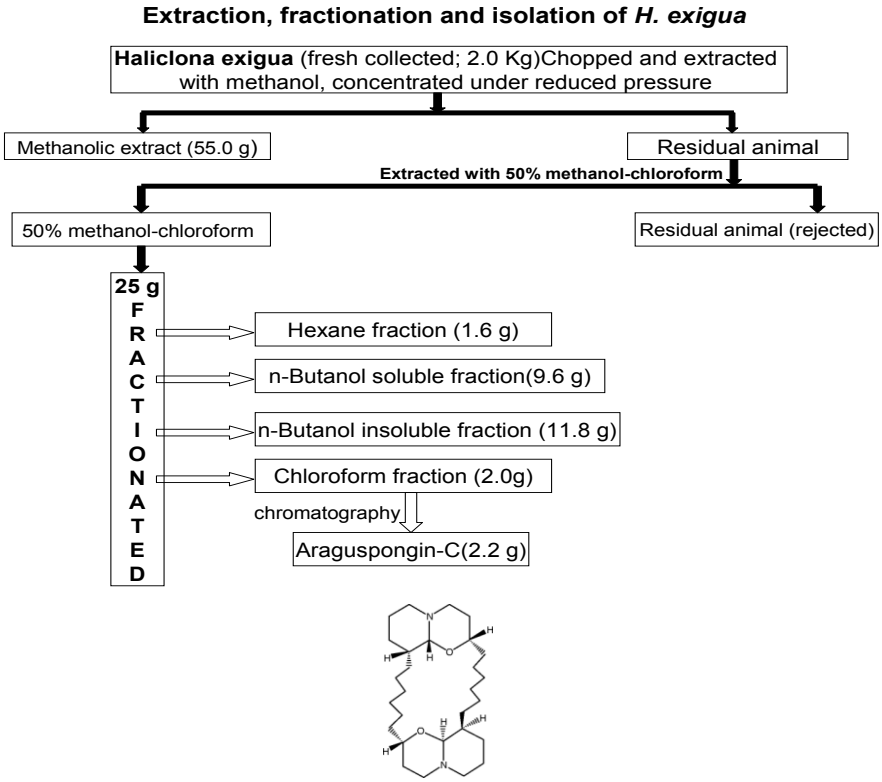

Fig. 2 Chemical structure of pure compound araguspongin C isolated from chloroform fraction of *H. exigua*

2.5 In vivo efficacy:

(i) Drug preparation

The sample was prepared as fine suspension in distilled water and the pure compound was pulverized to fine powder to make a fine suspension in distilled water with the help of 0.1% Tween-80.

(ii) Treatment schedule:

The methanol extract of *Haliclona exigua* was administered for 5 consecutive days at 500 mg/ kg body weight, orally, while the active fraction i.e. chloroform fraction was fed orally at 250 mg/kg for 5 days. The standard drug DEC was also fed orally at 50 mg/kg body weight for a better comparison. The sample solutions/suspensions were freshly prepared daily just before administration to infected animals.

(iii) Assessment of antifilarial efficacy

Micro- and macrofilaricidal efficacy were evaluated as described earlier (Misra-Bhattacharya et al. 2004). Mastomys infected 5-8 months back showing progressive rise in microfilaraemia were selected for the treatment. Blood smears of 10 µl tail blood were made just before the initiation of treatment i.e. on day 0 and after infection on days 8 and 15, and thereafter at fortnightly intervals till day 90. Any change in mf count in comparison to pretreatment level was expressed as percent change in microfilaraemia from the pretreatment level and reduction in microfilaraemia with 8-15 days of initiation of treatment was considered as microfilaricidal efficacy. Animals infected under identical conditions received vehicle only to serve as controls. At the end of the observation period (on day 91) the treated and control *M. coucha* were euthanized and various tissues (lungs,

heart, testes, lymph nodes) 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, cell adherence on their surfaces, 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 microfilariae were considered as a sterilization effect of the extract on the female and percent sterile females were assessed (Misra et al. 1984).

(iv) Statistical analysis

The analysis of data was carried out by PRISM 3.0 using one way ANNOVA (nonparametric) and Dunnett's multiple comparison test.

3. Results:

(i) *In vitro* activity on adult *B. malayi*:

The crude methanol extract was found effective in killing adult *B. malayi* worms at 31.25 ug/ml concentration (both in motility and MTT assay) when tested at various concentrations (500-15.6 µg/ml). Of the four fractions isolated from methanol extract viz. hexane, chloroform, n-butanol soluble and n-butanol insoluble, the chloroform fraction was most active and killed adult *B. malayi* at a low conc. of 15.6 µg/ml and n-butanol soluble fraction killed adult worm at 31.25 µg/ml followed by other fractions. n-Hexane fraction was, however, completely inactive (Table-1).

Extracts/fractions/pure compounds	Conc. (µg/ml)	Antifilarial activity (µg/ml; in motility and MTT assay)
MeOH extract	500–15.6	LC100 31.25
Hexane fraction	500–15.6	Inactive
Chloroform fraction	500–15.6	LC100=15.6
n-Butanol-soluble fraction	500–31.25	LC100=31.25
n-Butanol-insoluble fraction	500–62.5	LC100=125
Pure compound (araguspongin C)	500–15.6	LC100=15.6
Ivermectin	–	LC100=10
No compound	–	–

Table 1. In vitro activity of crude methanol extract and fractions/pure compounds against adult female *Brugia malayi*

A pure compound araguspongin C isolated from the active chloroform fraction also killed adult worm *in vitro* at a low conc. of 15.6µg/ml.

(ii) *In vivo* microfilaricidal (MIF) activity:

Both the methanol extract and the active chloroform fraction did not show significant microfilaricidal actions in contrast to DEC on 8 day. However, microfilarial density in both the treated groups was much lower than those of untreated group and at some points of time (day 30, 75, 90) the decrease was statistically significant when compared to that of control. The untreated control animals on the other hand demonstrated a steady increase in microfilaraemia.

(iii) Macrofilaricidal activity:

Both methanol extract and the chloroform fraction revealed some degree of antifilarial activity on adult parasites of *B. malayi*. The crude methanol extract and DEC exerted 39.5± 6.28 % and 42.4±7.7% adulticidal action respectively while the chloroform fraction demonstrated 50.2±3.6% reduction in adult worm burden over untreated control(Table-3).

In addition, there was moderate effect on the reproductive potential of female worms in case of both crude extract and the fraction. The crude product and DEC resulted in to 46.5±13.03 % and 38.5±9.5 % embryostatic action respectively while the chloroform fraction caused sterility in 58.6±5.3 % of the recovered females (Table-3).
Thus it may be concluded that the crude methanol extract of marine sponge *H. exigua* is active against adult *B. malayi in vitro* and its activity resided in the chloroform fraction which showed marginally superior activity than the crude extract at lower dose. The extract and active fraction also demonstrated *in vivo* adulticidal and embryostatic action on *B. malayi* in the rodent model, *Mastomys coucha* when administered orally at 500 and 250 mg/kg, respectively for 5 consecutive days.

Extract/fraction	Dose (mg/kg, p.o.x 5days)	Animal nos.	% change in Mf/10 µl tail blood at various time periods (days) posttreatment (over day 0 pretreatment level)						
			8	15	30	45	60	75	90
MeOH extract	500	5	−35.8±53.4	−81.8±84.1	−119.2*±57.2	−101.5±85.3	−151.2±110.7	−147.9**±38.6	−117.1*±22.1
Chloroform fraction	250	5	−7.7±14.6	+42.4±12.8	+68.3±21.7	+116.0±41.5	+189.6±52.0	+189.6*±48.7	+265.8*±52.3
DEC	50	5	−79.0*±5.5	−74.7±7.4	−15.3*±37.3	−82.7**±82.6	+27.9**±46.9	+210.9*±138.0	+116.3*±71.7
Control	–	4	+37.8±12.68	+87.5±19.9	+205.5±50.8	+304.8±60.0	+460.0±98.5	+717.6±181.2	+1,061.0±378.1

*$P<0.05$ low significance between each treated group vs. control; **$P<0.01$ high significance between each treated group vs. control

Table 2. Effect on crude methanol extract and fractions on microfilaracmia in *B. malayi* infected *M. coucha*

Extract/fraction	Dose (mg/kg, p.o. × 5days)	Animal nos.	% reduction in worm recovery and sterilization over control			
			Male	Female	Total	% females sterile
MeOH extract	500	5	47.5±13.4	34.4±3.9	39.5±6.3**	46.5±13.0*
Chloroform fraction	250	5	50.0±8.8	50.4±2.9	50.2±3.6**	58.67±5.3**
DEC only	50	5	60.0±4.7	31.2±10.6	42.4±7.7**	38.5±9.5*

The reduction in worm recovery has been assessed by comparing the mean values of treated group with that of control group. Statistical analysis was done by comparing each treated group vs. common control using one-way ANOVA

*$P<0.05$, i.e., low significance; **$P<0.01$, high significance

Table 3. x Macrofilaricidal (adulticidal) activities of crude methanol extract and chloroform fraction of *H. exigua* against *B. malayi* in *M. coucha* and comparison with standard filaricide DEC

Discussion:

India is rich in medicinal plants and marine flora/ fauna. We have been engaged in the development of drugs from natural products through bioassay-guided extraction, fractionation, and isolation of active constituents. We have recently reported the antifilarial efficacy in a few marine flora and fauna against human lymphatic filarid *B. malayi* maintained in experimental animals (Lakshmi et al. 2004a, b). In recent years, marine natural product research has led to the development of number of candidate drugs (Fournet and Munoz 2002; Kayser et al. 2003). Recent reports indicate that several agents from marine source have shown efficacy against various ailments and are currently being studied for their preclinical or early clinical development (Haefner 2003). Out of the many antiparasitic marine products only a few may aspire to reach the clinical trial stage. Compounds isolated from a number of marine flora/fauna e.g. cone snails, corals, sponges, sea squirts, marine worms, bryozoans, sea slug, sharks etc. have been used in treating fungal infection, tuberculosis, malaria, nematode infection, bacterial/viral infection, pain management, cancer, and inflammation (Yan 2004). Diverse biological

activities has been demonstrated in marine sponges and their compounds (Reddy & Faulkner, 1997; Rangel and Dagger 1997, Lakshmi et al, 2004*;*Venkateswarlu et al., 1994; 1996). Manzamines, unique β-carboline alkaloids, first reported from the genus *Haliclona* exhibited a diverse range of bioactivities, including cytotoxicity, insecticidal, antibacterial, anti-inflammatory, anti-infective, antiparasitic, as well as curative activity against malaria in animal models (Rao et al. 2003, 2004*;* 2006). Dube et al., 2007 very recently reported on the antileishmanial activity of *H. exigua* which was located in the n-butanol soluble fraction. In the present study, antifilarial activity could be located in the chloroform fraction (LC=15.6) which was better than n-butanol soluble fraction (LC=31.25). The pure compound Araguspongin-C was isolated from the chloroform fraction and also showed excellent adulticidal action *in vitro* on *B. malayi*. The present findings thus reveal better adulticidal efficacy of the Chloroform fraction than the parent methanol extract of *H. exigua*. The microfilarial densities in both the treated groups were much lower than those of untreated group and at some points of time (day 30, 75, 90) the decrease were statistically significant when compared to that of control. In addition, both the extracts also exerted adverse effects on the reproductive potential of female worms with the chloroform fraction exerting comparatively higher embryostatic activity.

DEC a reference drug was also administered orally at 50 mg/kg body weight for 5 consecutive days in a group of 5 infected mastomys and the efficacy of DEC was determined simultaneously. DEC was used as a standard microfilaricidal drug which brought about 78.96±5.5% fall in the microfilarial count on day 8 as compared to insignificant microfilarial decrease by the crude extract and it's chloroform fraction (Table-2). The macrofilaricidal and embryostatic activity shown by chloroform fraction MAF=50.2±3.6% and 58.0±11.3% respectively was marginally higher than the crude extract or DEC (Table-3). Since DEC is known to be inactive against adult *B. malayi in vitro*, we used another standard filaricide ivermectin which is active in vitro on both microfilariae and adults of B. malayi.

For evaluating antifilarial activity, we employed screening based on motility and MTT assays (*in vitro*). MTT -based assay provides a reliable measure of drug-induced inhibitory effects on adults resulting in a dynamic picture of the responses of filaria parasites to the test sample. The antifilarial activity of the crude extract of *H. exigua* and its fraction *in vivo* was validated in *Mastomys coucha* infected with L3 stage of *B. malayi*. *Mastomys coucha* infected with sub-periodic *B. malayi* is being used world-wide as an excellent experimental rodent model for evaluating the antifilarial activity of synthetic and natural products (Sanger et al 1981; Zahner et al 1988, 2001a, b; Schares et al 1994; Bajpai et al, 2004; 2005a, b). Antifilarial activity (macro and microfilaricidal) of the crude extract and its chloroform fraction was observed on experimental animals at the doses used. Since both the crude methanol extract (LC-31.25) and chloroform fraction (LC=15.6) were effective *in vitro*, it was selected for *in vivo* evaluation in *B. malayi* mastomys model. 250 mg/kg x 5, p.o. of chloroform fraction was found to be superior in antifilarial activity as compared to 500 mg/kg × 5, p.o. of the crude methanol extract, indicating that the antifilarial principles of this sponge are present in higher quantity in this fraction. So, it was decided to isolate the pure chemical entity responsible for this activity. For this purpose, chloroform fraction was subjected to chromatographic separation, and several pure compounds were isolated. One alkaloid identical to araguspongin C was purified and characterized on the basis of its spectro-chemical data, namely, ^{1}H nuclear magnetic resonance, mass spectrometry, and infrared and ultraviolet spectroscopies, and the data were compared with those reported in the literature, thus confirming its identity. Araguspongin C showed high *in vitro* activity. The *in vivo* studies

with the pure compounds were limited by low yield of the compounds. Nevertheless, efforts are being continued to isolate them in larger quantity or to synthesize them to increase their availability for *in vivo* activity assessments. Apart from this, we are in the process of isolating more compounds, even in comparatively small amounts, which might possess a superior antifilarial action. The activity of the crude extract and chloroform fraction could be due to the combined or synergistic effects of more than one component. The strong activity on adult parasites *in vitro* and in *B. malayi*/mastomys model combined with its natural origin makes this marine product worth pursuing as a potential agent against lymphatic filariasis. The present findings warrant the isolation and characterization of more pure compounds from this sponge and the evaluation of their *in vitro* and *in vivo* efficacy in addition to *in vivo* efficacy of Araguspongin C against *B. malayi* to locate the active principle/s.

Acknowledgments: The work was supported by the Department of Ocean Development, New Delhi, India. We thank Director, CDRI for encouragement and support and to Mr. H. R. Mishra, N. P. Mishra, A. K. Roy and R. N. Lal for technical assistance. The financial assistance in the form of fellowship by the Council of Scientific and Industrial Research (SM) is gratefully acknowledged. This manuscript bears **CDRI communication no. 7797**

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