

## Antiamoebic activity of marine sponge *Haliclona exigua* (Krikpatrick)

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### Article Info

Received: 3 September 2008

Accepted: 11 September 2008

Available Online: 22 September 2008

DOI: 10.3329/bjpp.v4i1.1083

#### Cite this article:

Lakshmi V, Saxena A, Mishra SK, Mishra M, Srivastava S, Ghoshal S. Antiamoebic activity of marine sponge *Haliclona exigua* (Krikpatrick). Bangladesh J Pharmacol. 2009; 4: 55-59.

### Abstract

The methanol and methanol-chloroform (1:1) extracts of the freshly collected *Haliclona exigua* showed minimum inhibitory concentration (MIC) of 125 and 250 µg/mL respectively in *in vitro* studies, but when both of these were tested *in vivo* in rats, only methanol-chloroform showed 80% inhibition of trophozoites at the dose of 900 mg/kg body weight against *Entamoeba histolytica*. Therefore only methanol-chloroform extract was further fractionated into four fractions (*n*-hexane, chloroform, *n*-butanol soluble and *n*-butanol insoluble fractions). Out of these, only *n*-hexane and *n*-butanol soluble fractions showed 80% inhibition of trophozoites at 900 mg/kg dose. Further the chromatography of the *n*-butanol fraction yielded araguspongic acid which showed promising results at different doses.

### Introduction

Human amoebiasis due to *Entamoeba histolytica* infection is mainly associated with morbidity thus affecting the quality of life and pace of developmental activities of countries with warm climatic conditions. A consistently high global incidence of this disease has been reported from surveys carried out at different intervals of time (Beltran, 1948; Stanley, 2003). This disease also possesses a challenge to our national health programs. A number of therapeutic agents possessing potent *in vitro* action against trophozoites of *E. histolytica* have been used to combat this disease. So far, these have been found to be too toxic or providing only symptomatic relief. Leads to obtain novel molecules with antiamoebic activity have been obtained from natural products, either terrestrial plants or marine organisms. The scope of natural products have widened with the inclusion of marine biota. Sponges are known as a rich source of sesquiterpenes and diterpenes and alkaloids.

Drug from marine resources is an area which offers an unprecedented opportunity for their pharmacological exploration and hence has received great attention during recent years for natural product chemistry, a promising new area of study. Secondary metabolites

produced in marine organisms could be the source of bioactive substance and useful in modeling compounds for drugs (Faulkner, 2001; Haefner, 2003). Marine micro-organisms, whose immense genetic and biochemical diversity is only beginning, likely to become a rich source of novel chemical entities for the discovery of more effective drugs. Marine sponges are shown to exhibit antibacterial, insecticidal, antiviral and antiplasmodial activities (Rao et al., 2003; Yan, 2004). Antifungal activity of *Haliclona* spp. against *Aspergillus* strains has also been reported (Bhosale et al., 1999). Some marine sponges are reported to possess anti-leishmanial activity. These include *Amphimedon viridis*, *Acanthostrongylophora* sp., *Neopetrosia* sp., *Plakortis angulospiculatus*, and *Pachymatisma johnstonii* (Rangel and Dagger, 1997; Le Pape et al., 2000; Marchan et al., 2000; Nakao et al., 2004; Compagnone et al., 1998; Copp et al., 2003; Rao et al., 2003; Rao et al., 2004). However, the number of antiamoebic compounds isolated from marine resources is still limited. Few researchers tried to isolate the chemical constituent of the *Haliclona exigua* (Reddy and Faulkner, 1997; Venkateshwarlu et al., 1994). The activity reported by the various workers in this sponge inhibited the rat brain nitric oxide synthase activity (Venkateshwara et al., 1998).



The present communication deals with the amoebicidal activity *H. exigua*, a marine sponge, against trophozoites of *E. histolytica* both *in vitro* and against experimental cecal amebiasis of rats.

*H. exigua* (Krikpatrick) belongs to Phylum Porifera, Class- Demospongia, Order- Haploscleridae, Family- Haliclioniidae. Animal colonies, sedentary, brownish yellow, asymmetrical 4 to 10 cm. irregularly rounded, amorphous in nature, remain attached to sea bottom by means of masses of spicules and possess the corn type of canal system. These colonies are attached on the dead coral stones in shallow water areas at the depth of 3-6 m in subtidal region. These sponges are found in Vallai Island, Setukarai, Gulf of Mannar, Ramnathpuram and Tamil Nadu Coasts of India.

## Materials and Methods

### Collection of material

*H. exigua* (Kirkpatrick) was collected from Tamil Nadu coast of India in the month of August, 1999. Specimen sample (Voucher specimen No. 343) has been preserved in the herbarium of Botany Division, Central Drug Research Institute, Lucknow, India. Fresh sponges were filled in the steel containers containing *n*-butanol on Tamil Nadu coast of India and were transported to CDRI laboratory.

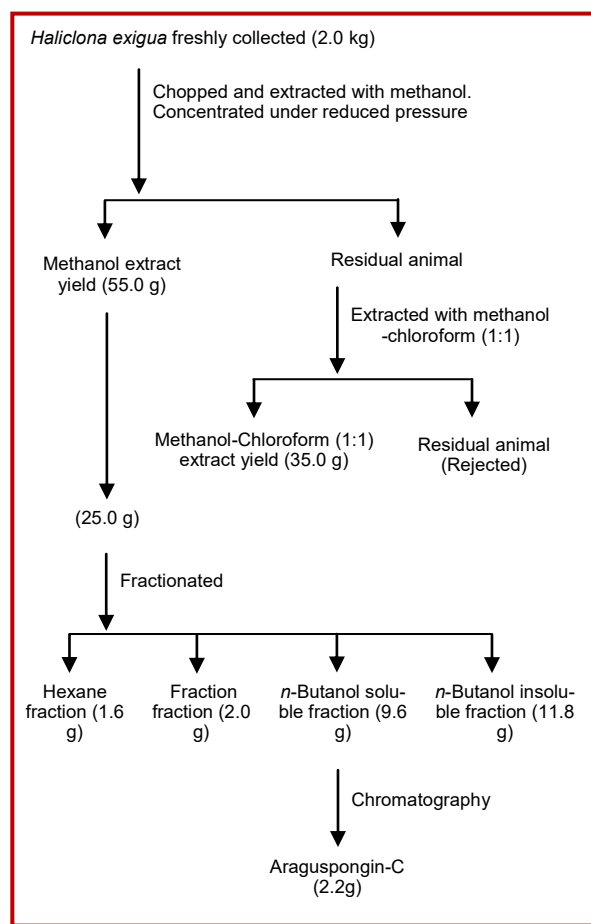
### Extraction/fractionation/isolation procedures

*H. exigua* (2 kg fresh weight) were chopped into small pieces and filled in glass containers soaked in methanol (5 x 3.0 L). The combined methanolic extract was concentrated under reduced pressure below 50°C into a viscous mass which was further dried under high vacuum (weight 55 g). The residual organism was further extracted with 3 L of methanol-chloroform (1:1) 5 times. The combined methanol-chloroform extract was further concentrated to the residual mass as in case of methanol extract. On complete drying the methanol-chloroform extract (35 g) was obtained. Since the methanol extract was found showing promising activity *in vivo* model, it was fractionated into four fractions *n*-hexane, chloroform, *n*-butanol soluble and *n*-butanol insoluble fractions and all the fractions were screened for antiamebic testing, the *n*-hexane and *n*-butanol soluble were found showing promising results. The *n*-hexane fraction was found to be a complex mixture and was of low yield therefore, *n*-butanol fraction was chromatographed over a column of silica gel and a pure compound was isolated and characterized as araguspongin-C with the help of spectroscopic data given in the literature for araguspongin-C, a known molecule isolated from the same sponge (Venkateshwarlu et al., 1994) and screened for the antiamebic activity, the other compounds present in the

extract could not be screened because of small amounts.

Araguspongin-C from *n*-butanol fraction showed very promising results at different doses executing 100% cures at 300 mg/kg.

It is important to note that the pure compound form *H. exigua* exhibited dose related curative activity against cecal amebiasis of rats. The rationale behind elevating the dose from 100 mg/kg to 300 mg/kg is, as compared to control rats with cecum which were shapeless with ulcers and mucoid contents the rats treated with 100 mg/kg of the pure compound, providing 20% cures, had cecum which were normal in size, shape and appearance. However, although the cecal wall and contents of all the treated rats were normal some amoebae were observed in the cecal smears. Hence, the dose was enhanced to 200 and 300 mg/kg for 5 days. At 200 mg/kg not much enhancement in activity was observed while at 300 mg/kg the activity elevated to 83.3%. In this group, the rats which were not cured had insignificant number of amoebae in the cecal smears



which, otherwise, had normal cecal wall and contents. When the time schedule of the rats receiving 300 mg/kg was increased by two days (7 days) a result of 100% was continuously obtained. The entire drug treated rats

exhibited cecum with normal contents comparable to the rats treated with standard drug metronidazole at 100 mg/kg for 5 days. Although the pure compound, araguspongin-C, reported in this study provided a complete cure at a higher dose schedule (300 mg/kg x 7 days) than the standard compound, it holds promise being identified from a marine organism, which thrives in nature.

#### Test models and methodology for antiamebic activity (in vitro model)

Axenic culture of *E. histolytica* (200: NIH) maintained TYI-S-33 medium (Diamond et al., 1978) has been used for *in vitro* screening. Xenic culture 2771 isolated from an acute case and maintained in Robinson's medium (Robinson, 1968.) was used to produce experimental cecal amebiasis in rats.

#### Evaluation of in vitro amebicidal activity

The stock solution of the test agent is prepared by adding small quantity of dimethylsulfoxide and required amount of water. Further serial double dilutions were prepared using triple glass distilled water. Amebic inoculum 0.1 mL containing approximately 2,000 trophozoites was added to the cavities of shallow culture slides to which the test sample (0.1 mL) in its

required dilution is added. Each cavity was then sealed with cover slip. The slides were kept in the moist chamber at 37°C. Observations were taken at 24 and 48 hours intervals. The activity of the test agent at the particular dilution was related with cent percent mortality. Metronidazole was the standard compound used. Duplicate sets were kept for each dilution (Das, 1975.).

#### Antiamebic in vivo test model method

Experimental production of cecal amebiasis of rats: Rats were fed on autoclaved rice diet for seven days prior infection. The cecal contents of these rats attain a pH of 5.5 to 7.0 without the occurrence of free ammonia which is toxic to these amoebae (Prasad and Bansal, 1983; Leitch, 1988) thus aiding in the consistent production of cecal infection. Rats under ether anesthesia were inoculated intracurally with 0.2 to 0.3 mL of amebic inoculum containing  $10 \times 10^4$  trophozoites of *E. histolytica* and the abdominal lesion sutured. After 48 hours the infected rats were ready for therapeutic evaluation of test agents as trophozoites of *E. histolytica* were visible microscopically in the contents and scrapings of the cecal wall. The animals were divided into two groups. One group was given oral administration of the drug, while the other group served as control group.

Table I

#### Antiamebic activity of *Haliclona exigua* against *E. histolytica* in *in vitro* and *in vivo* models

Name of the extracts/fractions/compounds	Antiamebic activity against <i>E. histolytica</i>		
	<i>In vitro</i> MIC (mg/mL)	<i>In vivo</i>	
		Dose mg/ kg (days)	% inhibition
Methanol extract	125	900 (5)	80
		500(5)	40
Methanol-chloroform (1:1) extract	250	900 (5)	40
		500 (5)	25
<i>n</i> -Hexane solution fraction from methanol extract	62.5	900 (5)	80
		500	50
Chloroform soluble fraction from the methanol extract	250	900 (5)	0
		500 (5)	0
<i>n</i> -Butanol soluble fraction of the methanol extract	125	900 (5)	80
		500 (5)	60
Aqueous fraction of the methanol extract	250	-	-
Araguspongin-C	250	100 (5)	20
		200 (5)	50
		300 (5)	80
		300 (5)	83.3
		300 (7)	100
		300 (7)	100
		300 (7)	100
Metronidazole (standard)	8	50 (5)	60
		100 (5)	100

### Treatment schedule

The test material was suspended in gum acacia suspension in distilled water. The rats were administered orally the test agent at 900 mg/kg with the help of a feeding needle once daily for five consecutive days. The rats were sacrificed 48 hours after the last dose of test material with an overdose of ether anesthesia and the cecum examined for trophozoites of *E. histolytica*. The reported method in literature (Neal, 1951) was used to evaluate the degree of infection.

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## Results and Discussion

The effect of *H. exigua* extracts on trophozoites of *E. histolytica* *in vitro* and against cecal amebiasis of rats is described in Table I. *In vitro* efficacy was recorded for all the test samples including the pure compounds. The *in vivo* therapeutic efficacy of the crude extracts showed that the methanol extract when administered at a dose of 900 mg/kg body weight for five days effected 80% cures. The *n*-butanol and *n*-hexane fractions of the same extract exhibited high efficacy with 80% cures at 900 mg/kg dose. Purified compound araguspongin-C from *n*-butanol fraction showed very promising results at different doses executing 100% cures at 300 mg/kg.

It is important to note that the pure compound form *H. exigua* exhibited dose related curative activity against cecal amebiasis of rats. The rationale behind elevating the dose from 100 to 300 mg/kg is, as compared to control rats with cecum which were shapeless with ulcers and mucoid contents the rats treated with 100 mg/kg of the pure compound, providing 20% cures, had cecum which were normal in size, shape and appearance. However, although the cecal wall and contents of all the treated rats were normal some amebae were observed in the cecal smears. Hence, the dose was enhanced to 200 and 300 mg/kg for 5 days. At 200 mg/kg not much enhancement in activity was observed while at 300 mg/kg the activity elevated to 83.3%. In this group, the rats which were not cured had insignificant number of amebae in the cecal smears which, otherwise, had normal cecal wall and contents. When the time schedule of the rats receiving 300 mg/kg was increased by two days (7 days) a result of 100% was continuously obtained. The entire drug treated rats exhibited cecum with normal contents comparable to the rats treated with standard drug metronidazole at 100 mg/kg for 5 days. Although the pure compound, araguspongin-C, reported in this study provided a complete cure at a higher dose schedule (300 mg/kg x 7 days) than the standard compound, it holds promise being identified from a marine organism, which thrives in nature.

It is not uncommon that marine organisms possess activity against pathogenic bacteria, fungus and proto-

zoa. Terpenoids isolated from *Pseudopleurauria wagnaari* possess antiamebic activity *in vitro*. Lobane diterpene derivatives of this organism were active against phytopathogenic fungus, *Cladosporium cucumerinum*, Gram positive bacteria, *Bacillus subtilis*, and yeast, *Saccharomyces cerevisia* (Edrada et al., 1998). Similar derivatives have been isolated from other marine organisms (Shin and Fenical, 1991). In view of the results presented it is evident that marine organisms can provide leads for antiamebic agents in future. Thus, the ocean with its innumerable biota offers a challenge to both chemists and biologists alike as it is a large reservoir of novel chemical entities with therapeutic potential for human use.

The results assumed significance when viewed regarding the condition of the cecal wall. The cecum of rats receiving the crude extract and the *n*-butanol fraction appeared normal with thin cecal wall comparable to the rats treated with the standard drug metronidazole (100 mg/kg body weight). However, the cecal contents of the rats treated with the test agents although being normal was slightly less formed as compared to the metronidazole treated rats. The results become still more interesting when the cecum of the treated rats are compared with the untreated rat cecum which is shapeless with ulcers on the walls and with mucous and very little fecal matter as contents.

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## Conclusion

*H. exigua* possesses significant amebicidal activity against *E. histolytica*. In the present study, the active constituent possessing 100% activity at 300 mg/kg dose for 7 days has been identified.

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## Acknowledgements

Ministry of Earth Sciences, Government of India, New Delhi is acknowledged for financial support. The authors wish to thank Mr. H.R. Mishra and Mr. N.P. Mishra for their technical support and Dr. M.N. Srivastava for collection of sponge.

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