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Antileishmanial potential of a marine sponge *Haliclona oculata* against experimental visceral leishmaniasis

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PEER REVIEW

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Comments

The manuscript shows possibility of exploration of more marine flora and fauna in order to locate the active principle for their antileishmanial potential. The obtained data indicate that marine sponge has the potential to provide new lead toward development of an effective antileishmanial agent and, hence, calls for more exhaustive studies for exploiting the vast world of marine resources to combat the scourge of several parasitic diseases.
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ABSTRACT

Objective: To evaluate the antileishmanial activity of a marine sponge *Haliclona oculata*.
Methods: The crude methanol extract was prepared from the freshly collected sponge and its three fractions were also prepared by maceration method. The antileishmanial activity of these extract and fractions was tested against *Leishmania donovani*.
Results: The antileishmanial activity was tested both *in vitro* and *in vivo*. The crude methanol extract exerted almost complete inhibition of promastigotes (81.0%±6.9%) and 78.8%± 5.2% inhibition of intracellular amastigotes at 100 μg/mL with IC_{50} values of 29.5 μg/mL and 40.6 μg/mL, respectively. The treatment of 500 mg/kg (*p.o.*) of the crude methanol extract for 5 d for *Leishmania donovani* infected hamsters resulted in 78.35%±10.20% inhibition of intracellular amastigotes. At a lower dose (250 mg/kg), it exhibited poor efficacy. Among the fractions, highest *in vitro* (>75%) and *in vivo* (84.3%±10.2%) antileishmanial activity was observed in *n*-chloroform fraction with IC_{50} values of 54.2 μg/mL and 61 μg/mL against promastigotes and intracellular amastigotes, respectively. Hexane fraction and *n*-butanol (both insoluble and soluble) fractions were found inactive *in vitro* and *in vivo*.
Conclusions: Our findings indicate that this marine sponge has the potential to provide new insight toward development of an effective antileishmanial agent and, hence, more exhaustive studies are needed for exploiting the vast marine resources of the world to combat the scourge of several parasitic diseases.

KEYWORDS

Antileishmanial activity, *In vitro* and *in vivo*, Marine sponge, *Haliclona oculata*, *Leishmania donovani*

1. Introduction

Leishmaniasis with a group of clinical manifestations caused by approximately 20 *Leishmania* species, has remained a major health problem in 88 tropical and subtropical countries of the world[1]. Visceral leishmaniasis (VL), also known as kala-azar, is the most severe in all the forms of leishmaniasis. It is caused by members of *Leishmania donovani* (*L. donovani*) complex, resulting in fever, hepatosplenomegaly, cachexia, and blood cytopenia. VL has also been emerging as an opportunistic infection in individuals carrying HIV[2]. The chemotherapy of VL has been undermined by resistance, variable efficacy, toxicity, parenteral

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administration, and requirement for long courses of administration. In India, sodium antimony gluconate is no longer useful as a drug because more than 64% of VL patients fail to respond or promptly relapse due to development of drug resistance in the parasite[3]. Alternative chemotherapeutic treatments with amphotericin B and its lipid formulation have severe limitations due to their toxic effect and prohibitive cost of treatment. There is an urgent need for new chemotherapeutic drugs for the treatment of these diseases, which mainly affect people in developing countries. With the recent emphasis of the World Health Organization on the development of antileishmanial agents from natural products, an urgent search for a drug derived from marine flora and fauna as well as from terrestrial plants has been initiated.

Exploiting drug from marine resources 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[4,5]. Marine microorganisms, whose immense genetic and biochemical diversity is beginning to be appreciated, are likely to become a rich source for the discovery of more effective drugs.

Marine sponges are shown to exhibit antibacterial, insecticidal, antiviral, and antiplasmodial activities[6,7]. Antifilarial activity of *Haliclona* species has also been reported[8].

Few chemical compounds have been reported from marine sponges[9-12]. Some marine sponges are reported to possess antileishmanial activity. These sponges include *Amphimedon viridis*, *Acanthostrongylophora* specie, *Neopetrosia* specie, *Plakortis angulospiculatus*, and *Pachymatisma johnstonii*[6,13-15]. However, the antileishmanial compounds isolated from marine resources is still limited.

We have earlier reported antileishmanial activity of a marine sponge, *Haliclona exigua* (Kirkpatrick) (*H. exigua*) of the Indian coast. This sponge is found in Vallai Island, Setukarai, Gulf of Mannar, Ramnathpuram and Tamil Nadu coasts of India. It is sedentary and remains attached to sea bottom by masses of spicules. Its colonies are asymmetrical, brownish yellow and ranges from 4 to 10 cm. These colonies are attached on the dead coral stones in shallow water areas at the depth of 3-6 m in subtidal region. In the present study, we have evaluated another species of *Haliclona*, *i.e. Haliclona oculata* (*H. oculata*) and its various fractions for their antileishmanial activity both *in vitro* as well as *in vivo*.

2. Material and methods

2.1. Collection of the sponge material

The *H. oculata*, a marine sponge, was collected from Kursadai Islands, Ramnathpuram Tamil Nadu in the month of February. Specimen sample (voucher specimen Number 476) has been preserved in the herbarium of the Botany Division, Central Drug Research Institute, Lucknow, India.

2.1. Extraction, fractionation and isolation procedure

Freshly collected sponge *H. oculata* (2 kg) was cut into small pieces and extracted with methanol (4×4 L) at room temperature. The combined extract was filtered and concentrated under reduced pressure below 45 °C in a rotavapor to a viscous mass (45 g). The residual was further extracted with 50% methanol-chloroform (4× 4 L) and the combined extract was filtered and concentrated under reduced pressure as above to a green viscous mass (35 g) and the residual was removed.

The methanol extract (25 g) was fractionated into hexane (1.6 g), chloroform (2.0 g), *n*-butanol soluble (9.6 g) and *n*-butanol insoluble fractions (11.8 g). The chloroform fraction (1.9 g) was fractionated into eight fractions by column chromatography. The sub-fraction showing potent antileishmanial activity was analysed by Liquid chromatography–mass spectrometry analysis to indentify the pure chemical entity responsible for this activity[16-18].

2.2. Parasite culture

The promastigotes of *L. donovani* (MHOM/80/Dd8) were grown in Roswell Park Memorial Institute-1640 medium supplemented with 10% heat-inactivated foetal bovine serum (Sigma, USA), 100 IU/mL penicillin (Sigma, USA) and 100 μg/mL streptomycin (Sigma, USA) at 26°C. Transgenic parasites expressing green fluorescent protein (GFP) were cultured in the presence of 100 μg/mL of geneticin sulphate (G418).

2.3. Macrophage cell line culture and maintenance

The adherent mouse macrophage cell line J774A.1 was maintained in Dulbecco's modified Eagle's medium (Sigma, USA) supplemented with 10% heat-inactivated fetal bovine serum, 100 IU/mL penicillin and 100 μg/mL streptomycin at 37 °C in 5% CO_2 in a humidified atmosphere.

2.4. Preparation of stock solutions and administration of test samples

For *in vitro* testing, extract and fractions were solubilized in minimum volume of dimethyl sulphoxide (DMSO) before adding to sterile Roswell Park Memorial Institute-1640+10% fetal calf serum and stocked at a concentration of 1 mg/mL. The final concentration of DMSO never exceeded 0.1% in test medium, a concentration that has no effect on the growth of parasites or macrophage cells. For *in vivo* testing, required quantity of the extract/fractions were weighed,

and a suspension was made in Tween 20 or 10% ethanol or saline water by crushing them in a pestle mortar, and final concentrations were made with sterile water. Test materials were administered as per standard procedures reported earlier[19].

2.5. Antipromastigote activity

Log phase transgenic GFP-expressing promastigotes (1×10^6 cells/mL) were put into 48-well tissue culture plates (CellStar), and different concentrations (100, 50, 25 and 10 μg/mL) of standard drugs as well as test samples were administered. Untreated cells served as control. Each assay was performed in triplicate. At different time intervals (48-72 h) after treatment, cells were removed, washed in phosphate buffer solution (PBS), and analyzed in a FACSCalibur flow cytometry (Becton Dickinson, USA) equipped with a 15 mV, 488 nm, air-cooled argon laser with excitation at 488 nm and emission at 515 nm. Ten thousand cells were acquired for each analysis. Multiparametric data were analyzed by Cell Quest software (Becton Dickinson). The inhibition of parasite growth was determined by comparing the fluorescence levels of drug treated parasites with that of untreated control parasites. Miltefosine was used as a reference drug.

2.6. Activity against intracellular amastigotes

J774A.1 macrophages were cultured in 24 well plates (CellStar), to a cell density of 10^5 cells/well and infected with late log phase transgenic GFP-expressing promastigotes at a multiplicity of infection of 10:1 (parasite/macrophage) and incubated at 37 °C in 5% CO_2 for 8-12 h. Wells were later washed thrice with incomplete medium to remove non phagocytosed parasites, and finally, the wells were supplemented with complete medium. At different time intervals (48-72 h) after treatment with the test samples (100, 50, 25 and 10 μg/mL), cells were removed, washed in PBS, and analyzed in a FACSCaliber flow cytometry (Becton Dickinson, USA) equipped with a 15 mV, 488 nm, air-cooled argon laser with excitation at 488 nm and emission at 515 nm. Ten thousand cells were acquired for each analysis. Multiparametric data were analyzed by Cell Quest software (Becton Dickinson). The inhibition of parasite growth was determined by comparing the fluorescence levels of treated parasites with that of untreated control parasites.

2.7. Cytotoxicity assay

In vitro cytotoxicity of *H. oculata* extract and fractions on J774A.1 macrophages was assessed by colorimetric 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide reduction assay. Cells (10^5 per milliliter) were incubated with 100 μg/mL of the test samples and incubated at 37 °C in 5% CO_2 for 72 h. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide at a final concentration of 400 μg/mL was added and incubated further for 3 h at 24 °C. Cells were centrifuged at 4500 r/min, and pellets were dissolved in DMSO before taking the absorbance at 540 nm. The mean percentage of post treatment viable cells was calculated relative to control, and results were expressed as the concentration inhibiting cell growth by 50% (IC_{50}).

2.8. Animal host

Laboratory-bred male golden hamsters (*Mesocricetus auratus*) weighing 45-50 g were used as the experimental host. They were housed in climatically controlled rooms and fed with standard rodent food pellet (Lipton Ltd, Bombay) and water *ad libitum*. Experiments on the animals were performed following the guidelines of Institutional Animal Ethics Committee of the CDRI.

Hamsters were infected intracardially with late log phase promastigotes of *L. donovani*. Briefly, promastigotes were harvested by centrifugation at 2800 r/min for 15 min at 4 °C, washed thrice with PBS and resuspended in PBS to a concentration of 1×10^8 per 0.1 mL. Hamsters were inoculated intracardially with promastigotes in 0.1 mL of PBS. Parasite burden was assessed on Day 20-Day 25 post-infection by performing splenic biopsies as described previously[19]. Once the infection was established with promastigote form, further passages in hamsters were carried out with splenic amastigotes. For this, animals carrying 40-60 d infection were autopsied, their spleen removed aseptically, homogenized in PBS and centrifuged at 900 r/min for 5 min at 4 °C to sieve out tissue debris. Supernatant was centrifuged at 2500 r/min for 10 min, pellet washed twice with PBS. Animals were infected intracardially with 1×10^7 amastigotes in 0.1 mL PBS. Animals carrying 25-30 d infection were employed for drug screening.

2.9. Leishmanicidal efficacy against experimental VL in golden hamsters

L. donovani-infected hamsters were administered with 500 mg/kg, 250mg/kg and 100mg/kg of test samples by oral route. Miltefosine, the reference drug, was administered orally at a dose of 40 mg/kg for 5 d. Splenic parasite loads in treated and untreated animals were determined on Day 7 post-treatment by performing splenic biopsies[19]. Impression smears were made on glass slides, air dried, fixed in absolute methanol, and stained with 10% Giemsa for 30 min. Slides were counted for the number of amastigotes under the oil immersion objective (100×) of a phase-contrast micro-scope[20]. Percent parasite inhibition in treated animals was calculated using the formula

$$\% \text{ inhibition} = \frac{\text{AT-100}}{\text{IT-TI}}$$

where AT is the actual number of amastigotes per 100 spleen cell nuclei in treated animals, IT is the initial number of amastigotes per

100 spleen cell nuclei in treated animals, and TI is the times increase in untreated control animals.

2.10. Statistical analysis

Two to three replicates were done in case where activity was observed for confirmation and results were expressed as mean± SD. Statistical analyses were performed using Sigma Stat (V2.03) software.

3. Results

Because the chloroform sub-fraction possesses better efficacy, it was decided to isolate the pure chemical entity responsible for this activity. For this purpose, chloroform sub-fraction was subjected to HPLC-MS analysis wherein the presence of four compounds *viz.* mimosamycin, xestospongin-C, xestospongin-D and araguspongin-C were identified.

3.1. Inhibitory effect of H. oculata on promastigotes

The effect of methanol extract, its fractions, and sub fraction was evaluated against promastigote form of transgenic *L. donovani* expressing GFP by flow cytometry. Methanol extract and chloroform fraction and its sub fraction were highly effective causing almost complete parasite death (IC_{50}=29.5, 54.2, 36.5 μg/mL respectively) as evidenced by drastic parasite inhibition at a concentration of 100 μg/mL (Table 1). At lower concentrations, there was still significant inhibition of promastigote growth. Hexane fraction was found inactive against promastigote at 100 and 50 μg/ mL. Hexane and *n*-butanol (insoluble and soluble) fractions were found to be inactive at 100 μg/mL and lower doses, as there was negligible decrease in fluorescence levels. The reference drug, miltefosine, exhibited complete inhibition of parasite multiplication (>90%; Table 1).

Table 1

In vitro antileishmanial activity of *H. oculata*.

Extract and fractions	Concentration (μg/mL)	Activity against promastigotes (%)	Activity against intracellular amastigotes (%)
Methanol extract	100	81.0±6.9	78.8±5.2
	50	67.6±7.4	53.2±6.4
	25	Inactive	Inactive
Hexane fraction of methanol extract	100	Inactive	Inactive
	50	Inactive	Inactive
Chloroform fraction of methanol extract	100	79.0±3.7	74.6±4.2
	50	Inactive	Inactive
Sub fraction of the active chloroform fraction	100	83.0±7.7	76.8±5.1
	50	57.2±8.4	52.2±4.0
	25	Inactive	Inactive
n-Butanol soluble fraction of methanol extract	100	Inactive	Inactive
	50	Inactive	Inactive
n-Butanol insoluble fraction of methanol extract	100	Inactive	Inactive
	50	Inactive	Inactive
Meltifosine	40	96.0±2.7	90.3±4.7

3.2. Inhibitory effect of H. oculata on intra-macrophage amastigotes

The crude extract and its fractions were evaluated for their leishmanicidal effect on intracellular amastigotes. Methanol extract showed significant inhibition of intracellular amastigotes (78.8%± 5.2%) at a dose of 100 μg/mL (Table 1), whereas at 50 μg/mL, the inhibitory effect was 53.2%±6.4%. Its IC_{50} was 40.6 μg/mL (Table 2) . Chloroform fraction resulted in 74.6%±4.2% inhibition of amastigotes growth at 100 μg/mL but was weakly active or inactive at lower doses. Similarly the subfraction of the active chloroform fraction exhibited 76.8%±5.1% and 52.2%±4.0% inhibition at the dose of 100 μg/mL and 50 μg/mL respectively. The hexane and *n*-butanol fraction was inactive at the doses of 100 and 50 μg/mL, respectively.

Table 2

IC_{50} values (μg/mL) of extract and fractions against promastigote and intracellular amastigotes.

Extracts/fractions	Promastigotes	Intracellular amastigote
Methanol extract	29.5	40.6
Chloroform fraction of methanol extract	54.2	61.0
Sub fraction of the active chloroform fraction	36.5	42.3

3.3. Cytotoxic activities of H. oculata

Crude methanol extract showed a cytotoxic effect against J774A.1 cells with IC_{50} value of 34.6 μg/mL, whereas the extracts and pure compound were devoid of any cytotoxic effect (IC_{50}>100 μg/mL).

3.4. Antileishmanial activity of H. oculata in hamsters

Efficacy of the crude extract as well as its fractions was assessed against *L. donovani* infection in hamster model of VL. Methanol extract was found to be active and considerably good efficacy (78.35%±10.20%) was observed at the dose level of 500 mg/kg for 5 d. At 250 mg/kg for 5 d, it was inactive. Administration of chloroform fraction at a dose of 250 mg/kg for 5 d, *p.o.*, resulted in 84.3%±10.2% inhibition (Table 3) of *L. donovani* but inactive at the dose of 100 mg/kg for 5 d. Similarly the subfraction of the active chloroform fraction was active and showed 78.35%±6.20% inhibition. At doses of 100 mg/kg for 5 d, it was found to be poor active (55.0%±2.2% inhibition), whereas hexane and *n*-butanol (insoluble and soluble) fractions were inactive (<50% parasite inhibition) at similar dose levels. Miltefosine was highly effective causing 93.6%±6.3% inhibition of *L. donovani* at 40 mg/kg for 5 d, *p.o.*

Table 3
In vivo antileishmanial activity of *H. oculata*.

Extracts/Fractions	Dose (mg/kg)	% Suppression
Methanol extract	500	78.35±10.20
Chloroform fraction of methanol extract	250	84.30±10.20
	100	Inactive
Sub fraction of the active chloroform fraction	250	78.35±6.20
	100	55.00±2.20
Meltifosine	40	93.60±6.30

4. Discussion

There are many marine products possessing antiparasitic activity, but only few have the hope to reach the clinical trial stage. Some of the marine natural compounds, isolated from cone snails, corals, sponges, sea squirts, marine worms, bryozoans, sea slug, and sharks were used for treating fungal infection, tuberculosis, nematode infection, malarial infection, bacterial infection, viral infection, pain management, cancer, and inflammation control. Few marine sponges are reported to exhibit diverse range of bioactivities including cytotoxicity, insecticidal, antibacterial, anti-inflammatory, anti-infective, and antiparasitic, as well as the exciting curative activity against malaria in animal models[6,15]. For example, illimaquinone, isolated from marine sponge, has been shown to have an antiproliferative effect against *Leishmania*. Similarly, manzamines, unique β-carboline alkaloids, were first reported from the genus *Haliclona*. We also have demonstrated considerably good antileishmanial activity, though, in the crude extract of *H. exigua* and its fraction only. The pure compound was ineffective. In the process, we thought it is worthwhile to explore the potential of another species of *Haliclona i.e. H. oculata* for its antileishmanial activity.

In the present study, crude methanolic extract of *H. oculata* and its chloroform fraction as well as sub-fraction showed considerably good inhibitory activity against both promastigote and intracellular amastigote forms of *L. donovani* but was inferior to the activity of standard drug miltefosine. Activity in macrophage-amastigote system is encouraging because the morphology and biochemistry of the intracellular amastigotes is similar to that of the clinical amastigote stage. The other fractions like hexane fraction and *n*-butanol fraction (soluble and insoluble) were ineffective against both the stages.

The antileishmanial activity of the potential extract of *H. oculata i.e.* methanolic and its chloroform fraction and sub-fraction was validated in male golden hamsters infected with *L. donovani*. Hamsters have proven to be a good animal model for the study of human VL, as it presents a progressive disease similar to human kala-azar[21]. Methanolic extract exhibited good efficacy at 500 mg/kg. The infected animals tolerated the treatment with no drastic changes in body and organ weight in treated group as compared to the untreated group. The activity of chloroform fraction at the dose of 250 mg/kg for 5 d, *p.o.*, was found to be superior to the methanolic extract, indicating that the antileishmanial principles of this plant are present in higher quantity in this fraction. The activity of sub-fraction of chloroform fraction was equal to that of methanolic extract and its chloroform fraction.

In our earlier studies with *H. exigua* one alkaloid identical to araguspongin C was found to have moderate activity *in vitro* and was ineffective *in vivo*. These studies were limited by low yield of the compounds. Nevertheless the excellent activity of the chloroform sub-fraction could be due to the combined or synergistic effects of more than one component. The antileishmanial efficacy exerted in this study by *H. oculata* was not comparable to the potent action of miltefosine (standard drug); however, its effective action on *in vitro* stages and moderate action in hamsters combined with its natural origin makes this marine product worth pursuing as a potential agent against leishmaniasis. The present findings warrant the exploration of more marine flora and fauna in order to locate the active principle for their antileishmanial potential.

Conflict of interest statement

We declare that we have no conflict of interest.

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Comments

Background

Leishmaniasis is a dangerous parasitic disease which is spread by the bite of phlebotomine sand flies. Conventional methods for treatment of leishmaniasis are not always effective, therefore the development of new methods based on the use of natural sources is of a priority and highly relevant.

Research frontiers

The present manuscript depicts antileishmanial activity of extracts of marine sponge species *H. oculata* and its various fractions both *in vitro* as well as *in vivo*. Authors demonstrate that methanol extract and chloroform fractions and its sub fraction were highly effective causing almost complete parasite killing as evidenced by parasite inhibition.

Related reports

Marine sponges are shown to exhibit antibacterial, insecticidal, antiviral, and antiplasmodial activities. Antifilarial activity of *Haliclona* species has also been reported. Few chemical compounds have been reported from marine sponges. Some marine sponges are reported to possess antileishmanial activity.

Innovations and breakthroughs

The antileishmanial efficacy exerted in this study by *H. oculata* was not comparable to the potent action of miltefosine (standard drug); however, its effective action on *in vitro* stages and moderate action in hamsters combined with its natural origin makes this marine product worth pursuing as a potential agent against leishmaniasis.

Applications

The present findings warrant the exploration of more marine flora and fauna in order to locate the active principle for their antileishmanial potential.

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The manuscript shows possibility of exploration of more marine flora and fauna in order to locate the active principle for their antileishmanial potential. The obtained data indicate that marine sponge has the potential to provide new lead toward development of an effective antileishmanial agent and, hence, calls for more exhaustive studies for exploiting the vast world of marine resources to combat the scourge of several parasitic diseases.

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