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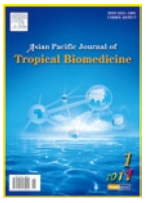

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In vitro antiplasmodial activity of marine sponge *Hyattella intestinalis* associated bacteria against *Plasmodium falciparum*

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ABSTRACT

Objective: To identify the antiplasmodial drugs from the marine sponge *Hyattella intestinalis* (*H. intestinalis*) associated bacteria. **Methods:** The *H. intestinalis* samples were collected from Thondi coast and subjected for enumeration and isolation of associated bacteria. Filter sterilized extracts (100, 50, 25, 12.5, 6.25 and 3.125 μg/mL) from bacterial isolates were screened for antiplasmodial activity against *P. falciparum* and potential extracts were also screened for biochemical constituents. **Results:** The count of THB isolates were maximum in November 2007 (20×10^4 CFU/g) and the average count was maximum during the monsoon season (77×10^3 CFU/g). A total of 29 bacteria were isolated based on the morphological characteristics and screened for antiplasmodial activity. The antiplasmodial activity of THB20 extract (IC_{50} 41.88 μg/mL) showed at two fold concentration of IC_{50} value of the positive control chloroquine (IC_{50} 19.59 μg/mL) and 14 bacterial isolates showed IC_{50} value of more than 100 μg/mL. Statistical analysis reveals that, significant *in vitro* antiplasmodial activity ($P<0.05$) was observed between the concentrations and time of exposure. The chemical injury to erythrocytes showed no morphological changes in erythrocytes by the ethyl acetate extract of THB isolates after 48 h of incubation. The antiplasmodial activity of potential bacterial isolates might be due to the presence of sugars and alkaloids in the ethyl acetate extracts. **Conclusions:** It is concluded from the present study that, the ethyl acetate extracts of THB20 posses novel metabolites for the development of newer antiplasmodial drugs.

1. Introduction

Natural products have been the source of most of the active ingredients of medicines. More than 80% of drug substances were natural products or inspired by a natural compound. The comparisons of the information presented on sources of new drugs from 1981 to 2007[1,2] indicate that almost half of the drugs approved since 1994 are based on natural products. The marine environment is frequently recognized as the largest potential source of biodiversity and it is being increasingly searched for novel chemicals with useful bioactivity. Identification of potential drugs from living organisms particularly from marine organisms could solve several hurdles in the management of malaria. Among the marine organisms, marine animals have been identified as a good source for drug development. Due to lack of cultivation technology multiple years taken for the production of effective bioactive principles could not meet out the urgent demand of antimalarial drugs. Marine invertebrates have developed highly specific relationship with numerous associated microorganisms and these associations are of recognized ecological and biological importance[3]. It has been reported that, the ratio of microorganisms with antimalarial activity from invertebrates were higher than other sources[4], which suggest that invertebrate associated microorganisms might play a chemical defence role for their host. This kind of microorganisms as a sustainable resource has high potential to biosynthesis more biologically active secondary metabolites within a short span of time due to well developed cultivation technology. With the aim of finding new bioactive compounds from marine microorganisms, the present study has initiated to isolate sponge associated bacteria showing potential antiplasmodial activity distributed along the coast of Palk Strait region, South east coast of India.

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2. Material and methods

2.1. Isolation of sponge associated bacteria

Marine sponge *H. intestinalis* was collected by by-catch at Thondi (Latitude 9°44′ 9″ N and Longitude 79°1′ 12″ E) in the Palk Strait region of Tamil Nadu and was authenticated by Dr. S Lazarus, Emeritus Fellow (Retired), Centre for Marine Science and Technology, Manonmaniam Sundaranar University, Rajakkamangalam, Kanyakumari District, Tamil Nadu, India. All the collected samples were washed thrice with tap water and twice with distilled water to remove the adhering associated animals. One gram of sponge samples was cut into small pieces and serially diluted. Diluted sample was subjected for continuous shaking in a thermostat shaker and plated in triplicate on Zobell Marine agar 2216 medium (HiMedia Laboratories Pvt. Limited, Mumbai, India) using pour plate method. The plates were incubated in an inverted position for 24 h at (28±2) ℃ and the colonies were counted and recorded. Based on the morphological characteristics (forms, elevation, margin and colour of the colony), the colonies were selected and restreaked thrice in a nutrient agar medium (HiMedia Laboratories Pvt. Limited, Mumbai, India) and stored on nutrient agar slants.

2.2. Mass cultivation of total heterotrophic bacteria

A loopful inoculum of 29 bacterial isolates were further inoculated into 500 mL conical flask containing 100 mL of nutrient broth (pH 7.2) prepared with 50% of aged seawater and kept at (28±2) ℃ for 24 h with continuous shaking. Twenty milliliter of the broth culture was then transferred to 1 000 mL of nutrient broth prepared with 50% of aged seawater and incubated for 4-5 days under continuous shaking.

2.3. Extraction of bioactive principles from bacteria

The mass cultures of bacterial isolates were adjusted to pH 5.0 using 1 N hydrochloric acid and centrifuged at 3 000 rpm for 5 min to remove cells. The supernatant was collected and was mixed with equal volume of ethyl acetate in a separating funnel. After vigorous shaking, the flask was kept undisturbed until two separate layers obtained (aqueous and organic). The upper organic phase was concentrated in a vacuum evaporator at 40 ℃ for 24 h and the crude extract was obtained. This process was repeated three times to obtain complete extraction of active principles.

2.4. Parasite cultivation

The antiplasmodial activity of isolated bacterial extracts was assessed against *Plasmodium falciparum* (*P. falciparum*) obtained from the Jawaharlal Nehru Centre for Advanced Scientific Research, Indian Institute of Science, Bangalore, India. *P. falciparum* are cultivated in human O Rh^+ red blood cells using RPMI 1640 medium (HiMedia Laboratories Private Limited, Mumbai, India)[5] supplemented with O Rh^+ serum (10%), 5% sodium bicarbonate (HiMedia Laboratories Private Limited, Mumbai, India) and 40 μg/mL of gentamycin sulphate (HiMedia Laboratories Private Limited, Mumbai, India). Hematocrits were adjusted at 5% and parasite cultures were used when they exhibited 2% parasitaemia[6].

2.5. In vitro antiplasmodial assay

Filter sterilized extracts (100, 50, 25, 12.5, 6.25 and 3.125 μg/mL) from 29 bacterial isolates were incorporated into 96 well tissue culture plates containing 200 μL of *P. falciparum* culture with fresh red blood cells diluted to 2% hematocrit. Negative control was maintained with fresh red blood cells and 2% parasitized *P. falciparum* diluted to 2% hematocrit, positive control was maintained with parasitized blood cells culture treated with chloroquine and artemether[7]. Parasitaemia was evaluated after 48 h by Giemsa stain and the average percentage suppression of parasitaemia was calculated by the following formula: Average % suppression of parasitaemia = Average % parasitaemia in control − Average % parasitaemia in test/Average % parasitaemia in control × 100.

2.6. Antiplasmodial activity calculation and analysis

The antiplasmodial activities of isolated bacteria were expressed by the inhibitory concentrations (IC_{50}) of the drug that induced a 50% reduction in parasitaemia compared to the control (100% parasitaemia). The IC_{50} values were calculated (concentration of extract in X axis and percentage of inhibition in Y axis) using Office XP (SDAS) software with linear regression equation. This activity was analyzed in accordance with the norms of antiplasmodial activity of Rasoanaivo *et al*[8]. According to this norms, an extract is very active if $IC_{50} < 5$ μg/mL, active 5 μg/mL < $IC_{50} < 50$ μg/mL, weakly active 50 μg/mL < $IC_{50} < 100$ μg/mL and inactive $IC_{50} > 100$ μg/mL.

2.7. Chemical injury to erythrocytes

To assess any chemical injury to erythrocytes that might be attributed to the extract, 200 μL of erythrocytes were incubated with 100 μg/mL of the extract at a dose equal to the highest used in the antiplasmodial assay. The conditions of the experiment were maintained as in the case of antiplasmodial assay. After 48 h of incubation, thin blood smears were stained with Giemsa stain and observed for morphological changes under high-power light microscopy. The morphological findings were compared with those in erythrocytes that were uninfected and not exposed to extract[9].

3. Results

The marine sponge *H. intestinalis* associated bacterial isolates counts is represented in Table 1. The count was maximum in the month of November 2007 (20×10^4 CFU/g) and minimum in the month of January 2007 and April 2008 (1×10^4 CFU/g). The average count was maximum during monsoon season (November − January) (77×10^3 CFU/g) and followed by summer season (May − July) (40×10^3 CFU/g).

A total of 29 different bacterial were isolated from *H. intestinalis* based on the morphological characteristics (Table 2). The THB20 extract (41.88 μg/mL) showed minimum level of IC_{50} value and followed by THB34 (42.36 μg/mL), THB33

Table 1
Counts of associated THB isolates from marine sponges *H. intestinalis*.

Sample No.	Month of collection	THB $\times 10^4$ CFU/g	Season	THB $\times 10^3$ CFU/g
1	August 2007	5	Pre monsoon	30
2	September 2007	2		
3	October 2007	2		
4	November 2007	20	Monsoon	77
5	December 2007	2		
6	January 2008	1		
7	February 2008	2	Post monsoon	23
8	March 2008	4		
9	April 2008	1		
10	May 2008	3	Summer	40
11	June 2008	2		
12	July 2008	7		

Table 2
Morphological characteristics and antiplasmodial IC_{50} values of bacterial isolates.

Strain No.	Form	Elevation	Margin	Colour of the colony	IC_{50} (μg/mL)
THB7	Circular	Convex	Entire	Light yellow	64.64
THB8	Circular	Convex	Entire	Yellowish	>100
THB9	Circular	Convex	Entire	Pearl white	51.56
THB19	Irregular	Raised	Undulate	Pale yellow	>100
THB20	Irregular	Flat	Curled	Light yellow	41.88
THB33	Circular	Convex	Entire	Yellow	46.23
THB34	Circular	Raised	Entire	Pale yellow	42.36
THB35	Circular	Raised	Entire	Waxy	80.63
THB46	Circular	Raised	Entire	Milky white	67.06
THB47	Circular	Convex	Entire	Light green	>100
THB48	Circular	Raised	Entire	Dull white	>100
THB59	Circular	Raised	Entire	Transparent white	>100
THB60	Circular	Raised	Entire	Transparent light yellow	94.37
THB71	Circular	Flat	Entire	Dark yellow	>100
THB72	Circular	Flat	Entire	Pink	>100
THB73	Circular	Raised	Entire	Waxy	66.58
THB86	Circular	Raised	Entire	Waxy	>100
THB87	Circular	Raised	Entire	Light yellow	>100
THB100	Circular	Raised	Entire	Light yellow	>100
THB101	Circular	Raised	Entire	Light orange	93.48
THB113	Circular	Raised	Entire	Light yellow	>100
THB114	Irregular	Raised	Undulate	Dull white	86.11
THB115	Irregular	Raised	Undulate	White	88.32
THB128	Circular	Raised	Entire	Dull white	48.01
THB129	Irregular	Raised	Lobate	Dull white	>100
THB139	Circular	Raised	Entire	Dull yellow	>100
THB140	Circular	Raised	Entire	White	75.34
THB149	Circular	Raised	Entire	Light brown	90.17
THB150	Irregular	Raised	Undulate	Waxy	>100
Positive control					
Chloroquine					19.59
Artemether					4.09

Values are found significant between concentrations and time of exposure ($P<0.05$).

(46.23 μg/mL) and THB128 (48.01 μg/mL). Among 29 bacterial extracts, 11 extracts showed IC_{50} values between 50 to 100 μg/mL and 14 extracts showed IC_{50} values more than 100 μg/mL (Table 2). The percentage of antiplasmodial activity IC_{50} values of *H. intestinalis* associated bacterial isolates against *P. falciparum* was presented in Figure 1.

The microscopic observation of uninfected erythrocytes added with the ethyl acetate extracts from bacterial isolates and uninfected erythrocytes from the blank column of the 96-well plate showed no morphological differences after 48 h of incubation. The analysis of preliminary biochemical constituents reveals that, the extracts from THB isolates have variety of biochemical constituents, namely alkaloids and reducing sugars (Table 3).

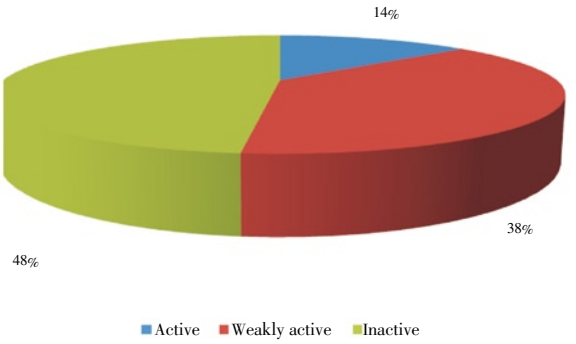

Figure 1. Percentage of antiplasmodial activity IC_{50} values of *H. intestinalis* associated bacterial isolates against *P. falciparum*.

Table 3
Biochemical constituents in chosen sponge associated bacterial isolates.

Biochemical constituents	THB20	THB33	THB34	THB128
Reducing sugars	+	+	+	−
Amino acids	−	−	−	−
Proteins	−	−	−	−
Alkaloids	+	+	+	+
Steroids	−	−	−	−
Triterpenoids	−	−	−	−

"+" indicates positive; "−" indicates negative.

4. Discussion

Malaria is a major tropical parasitic disease responsible for significant morbidity and mortality, *Plasmodium* species are responsible for malaria, an illness killing about 1–2 million people per year. Among the four types of human pathogenic parasites, *P. falciparum* is the most dangerous species. Due to the mounting prevalence of *P. falciparum* resistance to available antimalarial drugs such as chloroquine and artemisinin[10,11], the treatment of malaria is becoming more difficult. There is therefore an urgent need for the discovery of novel and efficient antimalarial drugs to treat malaria and to prevent the emergence of resistance.

Antiplasmodial drugs from marine halophytes have documented by our previous findings[12–14], but it shows side effects due to the presence of high concentration of tannins and phenols. And also keeping the biodiversity conservation of marine halophytes, the present study is mooted out to find out the possibility of developing antiplasmodial drugs from microorganisms. Friedrich *et al* estimated that, some sponge species have 40% of the animal biomass must be attributed by the bacteria[15], an amount that exceeds the bacterial population of seawater by two to four orders of magnitude[15]. The roles of sponge associated bacteria are reported to produce bioactive metabolites[16,17]. The earlier findings have encouraged the possibility of finding new antimalarial agents from sponge associated bacteria.

The present study has collected 12 sponge samples throughout the year at different seasons and all the samples have reported to harbour associated bacteria. This is due to the feeding process of sponges and bacteria from surrounding seawater are continuously swirled in by the sponge-driven currents and most of these bacteria are retained in the sponge body. Earlier investigation provided evidence that, the composition of the sponge inhabiting microbial consortium depends on the host species[18–22]. The average counts of *H. intestinalis* associated bacteria were varied in all four seasons. Thakur reported that, bacterial density in sponges is attributed to the temporal variations in the surrounding environment, and also linked to the irrigation system of the sponge. Previous studies proved that, some bacteria permanently reside in the sponge mesohyle, pointing to a close interaction between the host and associated bacteria[15,23].

The present study reveals that, THB20 and THB34 showed antiplasmodial IC_{50} value (41.88 μg/mL and 42.36 μg/mL respectively) of two fold IC_{50} value of positive control chloroquine. The antiplasmodial nature of sponge associated bacteria is due to the marine environmental conditions which are extremely different from terrestrial ones, it exposed to different pH, temperature, pressure, oxygen, light, nutrients and salinity. Earlier findings states that, the marine microorganisms showed potential antiplasmodial activity against *P. falciparum* [24–26]. According to Rasoanaivo *et al* 14%, 38% and 48% of extracts from bacterial isolates were classified as active, weakly active and inactive respectively[8].

The biochemical constituent analysis of potential extracts showed the presence of reducing sugars and alkaloids. Otoguro *et al* reported that, polysaccharides, polyketides and polysaccharide derivatives (Prumycin) are having potential antiplasmodial activity[27]. Stierle *et al* reported that, the presence of alkaloids and reducing sugars showed potential *in vitro* antiplasmodial activity[28–31]. The mode of action could be due to the inhibition of *P. falciparum* merozoites invasion into the erythrocytes[32] and disruption of *P. falciparum* rosettes[33] by the carbohydrates; inhibition of *P. falciparum* fatty acid biosynthesis[34], inhibition of hemozoin biocrystallization by the alkaloids[35]. Alkaloids are nitrogenous compounds derived from many biogenetic precursors and possessing antimalarial activity[36]. Numerous alkaloids isolated from marine sources have antimalarial activity[37,38]. These findings could encourage the marine microbes derived compounds for the antiplasmodial drug development. It is concluded from the present study that, the *H. intestinalis* associated bacterial isolates proved as an enormous source to come up with novel antiplasmodial drugs. Investigations are in progress to identify the active antiplasmodial compounds from *H. intestinalis* associated bacterial extracts by bioassay-guided fractionation.

Conflict of interest statement

We declare that we have no conflict of interest.

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