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In vitro antiplasmodial activity of *Clathria vulpina* sponge associated bacteria against *Plasmodium falciparum*

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ABSTRACT

Objective: To identify the possible antiplasmodial drugs from bacteria associated with marine sponge *Clathria vulpina* (*C. vulpina*). **Methods:** The *C. vulpina* samples were collected from Thondi coast and subjected to enumeration and isolation of associated bacteria. Filtered and sterilized extracts (100, 50, 25, 12.5, 6.25 and 3.125 μg/mL) from bacterial isolates were screened for antiplasmodial activity against *Plasmodium falciparum*. Potential extracts were also screened for biochemical constituents. **Results:** Thirty one bacterial isolates were isolated from twelve sponge samples collected from Thondi coast and screened for antiplasmodial assay. The count of bacterial strains were maximum in November 2007 (19×10^4 CFU/g) and the average count was maximum during the monsoon season (110×10^3 CFU/g). The antiplasmodial activity of isolate THB15 was highly comparable (IC_{50} = 20.73 μg/mL) with the positive control chloroquine (IC_{50} = 19.59 μg/mL) and 21 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 bacterial isolates after 48 h of incubation. The *in vitro* antiplasmodial activity might be due to the presence of sugars and alkaloids in the ethyl acetate extracts of bacterial isolates. **Conclusions:** The ethyl acetate extract of THB15 possesses lead compounds for the development of antiplasmodial drugs.

1. Introduction

Sponges are filter-feeding, sessile multicellular organisms that live mainly in marine habitats. They are an ecologically important and highly diverse component of marine benthic communities, with an estimated 15 000 species worldwide. Marine sponges harbor dense and diverse microbial communities, with many of the microorganisms being specific to sponge hosts[1-13]. There is direct evidence indicating that, compounds of potential pharmaceutical importance are actually derived from symbiotic bacteria rather than being produced by the sponge from which the particular compound was isolated[3,4]. This kind of bacteria act as a sustainable resource for the biosynthesis of more biologically active secondary metabolites within a short span of time due to well developed cultivation technology. With the aim to find out new bioactive compounds from marine microorganisms, the present study has initiated to isolate total heterotrophic bacteria showing potential antiplasmodial activity associated with the marine sponge *Clathria vulpina* (*C. vulpina*) distributed along the coast of Palk Strait.

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2. Material and methods

2.1. Isolation of sponge associated bacteria

Marine sponge *C. vulpina* was collected by by-catch at Thondi (Lat. 9° 44′ N and Lon. 79° 10′ 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 sterile aged seawater to remove the adhering

associated animals. One gram of sponge samples was cut into small pieces and serially diluted. Diluted sample was subjected to 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 isolated bacteria

A loopful inoculum of 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) ℃ with continuous shaking. Twenty milliliter of the broth culture was then transformed 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 ℃ and the crude extract was obtained. This process was repeated thrice 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, Bangalore, India. *P. falciparum* are cultivated in human O Rh^+ red blood cells using RPMI 1640 medium (HiMedia Laboratories Private Limited, Mumbai, India)[14] supplemented with 10% O Rh^+ serum, 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[15].

2.5. In vitro antiplasmodial assay

Filter sterilized extracts (100, 50, 25, 12.5, 6.25 and 3.125 μg/mL) from 31 bacterial isolates were incorporated into 96-well tissue culture plate 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[16]. 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[17]. This activity was analyzed in accordance with the norms of antiplasmodial activity of Rasoanaivo *et al*[18]. 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[19].

3. Results

The counts of bacterial isolates from *C. vulpina* sponge samples are represented in Table 1. The bacterial count was maximum in the month of November 2007 (19×10^4 CFU/g) and minimum in the month of April 2008 (1×10^4 CFU/g). The average count was maximum during the monsoon season (November − January) (110×10^3 CFU/g) and followed by summer season (May − July) (60×10^3 CFU/g). A total of 31 different bacterial isolates were isolated from *C. vulpina* based on the morphological characteristics. The extract of THB15, THB27 and THB2 showed IC_{50} value of less than 50 μg/mL. The extracts from THB1, THB3, THB16, THB28, THB29, THB68 and THB134 showed IC_{50} values between 50 to 100 μg/mL. Among 31 bacterial isolates extracts screened for antiplasmodial activity, 21 extracts showed IC_{50} values higher

Table 1
Counts of associated bacterial isolatess from marine sponges.

Sample No.	Month of collection	THB $\times 10^4$ CFU/g	Season	THB $\times 10^3$ CFU/g
1	August 2007	3	Pre monsoon	23
2	September 2007	2		
3	October 2007	2		
4	November 2007	19	Monsoon	110
5	December 2007	12		
6	January 2008	2		
7	February 2008	4	Post monsoon	23
8	March 2008	2		
9	April 2008	1		
10	May 2008	3	Summer	60
11	June 2008	8		
12	July 2008	7		

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

Isolate No.	Form	Elevation	Margin	Colour of the colony	IC_{50} (μg/mL)
THB 1	Circular	Convex	Entire	Light yellow with brown centre	54.31
THB2	Irregular	Umbonate	Lobate	Light yellow	37.58
THB3	Irregular	Raised	Undulate	Paper white	62.19
THB15	Rhizoid	Flat	Filamentous	Dull white	20.73
THB16	Circular	Convex	Entire	Light orange	70.06
THB27	Irregular	Raised	Undulate	Waxy	33.64
THB28	Circular	Raised	Entire	Waxy	88.77
THB29	Circular	Convex	Entire	Light yellow	70.06
THB41	Circular	Convex	Entire	Transparent yellow	>100
THB42	Circular	Convex	Entire	White	>100
THB43	Circular	Convex	Entire	Pink	>100
THB54	Irregular	Raised	Undulate	White	>100
THB55	Irregular	Raised	Undulate	Light brown colour	>100
THB66	Circular	Raised	Entire	Transparent white	>100
THB67	Irregular	Flat	Entire	Light yellow	>100
THB68	Circular	Flat	Entire	Light yellow	76.95
THB80	Circular	Flat	Entire	Dull white	>100
THB81	Circular	Flat	Entire	White	>100
THB82	Circular	Raised	Entire	White	>100
THB94	Circular	Raised	Entire	Light yellow	>100
THB95	Irregular	Flat	Undulate	White	>100
THB96	Circular	Raised	Entire	White	>100
THB108	Circular	Raised	Entire	Transparent yellow	>100
THB109	Irregular	Flat	Undulate	White	>100
THB122	Circular	Raised	Entire	Light yellow	>100
THB123	Circular	Raised	Entire	Transparent white	>100
THB124	Circular	Raised	Lobate	Waxy	>100
THB134	Circular	Raised	Entire	White	95.66
THB135	Circular	Convex	Entire	Yellow	>100
THB145	Circular	Raised	Entire	Dull white	>100
THB146	Circular	Raised	Entire	Dull white	>100
Chloroquine					19.59
Artemether					4.09

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

than 100 μg/mL (Table 2).

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 revealed that, the extracts from bacterial isolates have variety of biochemical constituents, namely alkaloids and sugars (Table 3).

Table 3
Biochemical constituents in chosen sponge associated bacterial isolates extracts.

Biochemical constituents	THB2	THB15	THB27
Reducing sugars	−	+	+
Amino acids	−	−	−
Proteins	−	−	−
Alkaloids	+	+	+
Steroids	−	−	−
Triterpenoids	−	−	−

+: Positive, −: Negative.

4. Discussion

Malaria is one of the most important health problems in tropical and subtropical countries. The World Health Organization estimates that, 2 300 million (41%) people of total world population have been living at high malaria risk. In 2009, 2.4 million parasitologically confirmed malaria cases and 3 320 deaths were reported in South-East Asia Region[20]. Marine invertebrates have developed highly specific relationship with numerous associated microorganisms and these associations are of recognized ecological and biological importance[3,7,8]. Unlike other invertebrates, sponges harbour extraneous microorganisms on their surface in their canal system and in the intracellular matrix which constitutes a large part of the body[21]. Earlier investigation reveals that, many compounds found in sponges are biosynthesized through microorganisms associated with them or indeed produced by microorganisms[4,5,22]. To confirm this hypothesis, there has been a great deal of interest in isolating bioactive microorganisms from sponges[23-28]. However, the emergence of strains of *P. falciparum* resistant to chloroquine and many other drugs in succession and many bioactive compounds isolated from sponge associated microbes has stimulated us to identify new antiplasmodial agents from the total heterotrophic bacteria associated with marine sponge *C. vulpina* distributed along the coast of Palk Strait.

The present study has collected 12 *C. vulpina* samples throughout the year at different seasons and all the sponge samples have been reported to harbour bacterial Isolates. Burja and Hill isolated 228 strains of bacteria, 25 fungi, 3 actinomycetes and 9 strains of cyanobacteria from sponge samples of Australian Great Barrier Reef[29]. The present study also found that, the bacterial isolates were maximum during the monsoon season (November - January). This might be due to the higher nutrient derived from the fresh water runoff from the adjacent river which supports the maximum growth of bacteria during rainy season. The present study observed that, THB15 showed antiplasmodial IC_{50} value of 20.73 μg/mL and the activity is comparable to the positive control chloroquine. According to Rasoanaivo *et al*[18] 3 bacterial extracts are active, 7 bacterial extracts are weakly active and 21 bacterial extracts are inactive in this study. Chinworrungsee *et al*[30-37] reported that halorosellinic acid showed antiplasmodial IC_{50} value of 13 μg/mL.

The potential extracts showed the presence of sugars and alkaloids. The mechanism of action may be due to the inhibition of *P. falciparum* merozoites invasion into the erythrocytes and disruption of *P. falciparum* rosettes by the carbohydrates, inhibition of *P. falciparum* fatty acid biosynthesis and inhibition of hemozoin biocrystallization by the alkaloids[38-41]. Stierle *et al*[42] reported that, the presence of alkaloids and reducing sugars showed potential *in vitro* antiplasmodial activity. The antiplasmodial activity of marine sponge associated microbial alkaloids (curcuphenol) has been reported by El Sayed *et al*[43]. Otoguro *et al*[44] reported that, polysaccharides, polyketides and polysaccharide derivatives (Prumycin) are having potential antiplasmodial activity. These findings could encourage the microbes derived compounds for the antiplasmodial drug development. It is concluded from the present study that, the *C. vulpina* associated bacterial isolates showed enormous resource to find out the new drugs with antiplasmodial activities. Investigations are in progress to identify the active antiplasmodial compounds of bacterial extracts by bioassay-guided fractionation.

Conflict of interest statement

We declare that we have no conflict of interest.

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