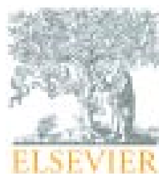

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# *In vitro* antiplasmodial activity of marine sponge *Stylissa carteri* associated bacteria against *Plasmodium falciparum*

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ABSTRACT

**Objective:** To identify the possible antiplasmodial drugs from bacteria associated with marine sponge *Stylissa carteri* (*S. carteri*). **Methods:** The *S. carteri* 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 $\mu$ g/mL) from isolated bacterial isolates were screened for antiplasmodial activity against *Plasmodium falciparum* (*P. falciparum*) and potential extracts were also screened for biochemical constituents. **Results:** Twelve samples of *S. carteri* were collected and subjected for enumeration and isolation of associated bacteria. The count of bacterial isolates were maximum in November 2007 ($34\times10^4$ CFU/g) and the average count was maximum during the monsoon season ($203\times10^3$ CFU/g). Thirty two morphologically different bacterial isolates were isolated from *S. carteri* and the ethyl acetate bacterial extracts were screened for antiplasmodial activity against *P. falciparum*. The antiplasmodial activity of a isolate THB17 ($IC_{50}$ 20.56 $\mu$ g/mL) extract is highly comparable with the positive control chloroquine ($IC_{50}$ 19.59 $\mu$ g/mL) and 13 bacterial extracts which showed $IC_{50}$ value of more than 100 $\mu$ g/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 reducing sugars and alkaloids in the ethyl acetate extracts of bacterial isolates. **Conclusions:** The ethyl acetate extract of THB17 possesses lead compounds for the development of antiplasmodial drugs.

## 1. Introduction

Natural products and their derivatives continue to play an important role in the development of drugs for the treatment of human diseases[1]. Marine invertebrates, such as sponges, have proven to be a rich source of biologically active and pharmacologically valuable natural products, with a high potential to become effective drugs for therapeutic use[2]. These marine sponges are the oldest metazoan group, having an outstanding importance as a living fossil[3]. These simplest animals are very efficient filter feeders. It has been estimated that, some of them are able to filter their own body volume of water every 5 seconds[4]. Earlier investigations reveals that, bacterial isolates associated with marine sponges had the ability to produce compounds that are similar and in some cases identical to those isolated from sponges[5,6]. The marine sponge associated bacteria are considered as a rich source of novel antiplasmodial agents and these potential resources were scarcely explored. The present study reported the findings of antiplasmodial potential of marine sponge *Stylissa carteri* (*S. carteri*) associated bacteria collected from the Palk Strait region, South east coast of India.

## 2. Materials and methods

### 2.1. Isolation of sponge associated bacteria

Marine sponge *S. carteri* was collected by by-catch at Thondi (Lat. 9°44′ 10″ N and Lon. 79°10′ 12″ E) in the Palk

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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 32 isolated 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 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 isolated 32 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 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)[7] 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[8].

### 2.5. *In vitro* antiplasmodial assay

Filter sterilized extracts (100, 50, 25, 12.5, 6.25 and 3.125 μg/mL) from 32 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[9]. 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*. According to this norms[10], 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[11].

## 3. Results

The counts of isolated bacterial isolates from *S. carteri* sponge samples are represented in Table 1. The bacterial count was maximum in the month of November 2007 ($34\times10^4$ CFU/g) and minimum in the month of August 2007, March 2008 and April 2008 ($1\times10^4$ CFU/g). The average count was maximum during monsoon season (November-January) ($203\times10^3$ CFU/g) and followed by summer season (May-July) ($43\times10^3$ CFU/g). A total of 32 different bacterial isolates were isolated from *S. carteri* based on the morphological characteristics (Table 2). The extract of THB17 (20.56 μg/mL) showed minimum level of $IC_{50}$ value and followed by THB5 (35.09 μg/mL) and THB31 (45.27 μg/mL). The extracts from THB4, THB44, THB56, THB57, THB58, THB83, THB97, THB98, THB99, THB111, THB125, THB136 and THB138 showed $IC_{50}$ values more than 100 μg/mL. Among 32 bacterial extracts screened for

antiplasmodial activity, THB6, THB18, THB30, THB32, THB45, THB69, THB70, THB84, THB85, THB110, THB112, THB126, THB127, THB137, THB147 and THB148 extracts showed $IC_{50}$ values between 50 to 100 μg/mL (Table 2). 9%, 50% and 41% of extracts from isolated bacterial isolates were classified as active, weakly active and inactive respectively.

**Table 1.**

Counts of associated bacterial isolates from marine sponges *S. carteri*.

| Month of collection | THB×$10^4$ CFU/g | Season | THB×$10^3$ CFU/g |
|---|---|---|---|
| August 2007 | 1 | Pre monsoon | 17 |
| September 2007 | 2 | | |
| October 2007 | 2 | | |
| November 2007 | 34 | Monsoon | 203 |
| December 2007 | 24 | | |
| January 2008 | 3 | | |
| February 2008 | 8 | Post monsoon | 33 |
| March 2008 | 1 | | |
| April 2008 | 1 | | |
| May 2008 | 2 | Summer | 43 |
| June 2008 | 9 | | |
| July 2008 | 2 | | |

The microscopic observation of uninfected erythrocytes added with the ethyl acetate extracts from bcaterial 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 bacteraial isolates have variety of biochemical constituents, namely alkaloids and reducing sugars (Table 3).

**Table 3.**

Biochemical constituents in chosen sponge associated bacterial isolates extracts.

| Name of the biochemical constituents | THB5 | THB17 | THB31 |
|---|---|---|---|
| Reducing sugars | + | + | – |
| Amino acids | – | – | – |
| Proteins | – | – | – |
| Alkaloids | + | + | + |
| Steroids | – | – | – |
| Triterpenoids | – | – | – |

"+" indicates positive "–" indicates negative

**Table 2.**

Morphological characteristics and antiplasmodial $IC_{50}$ values of isolated bacterial isolates.

| Isolate No. | Form | Elevation | Margin | Colour of the colony | $IC_{50}$ (μg/mL) |
|---|---|---|---|---|---|
| THB4 | Circular | Raised | Entire | Brownish yellow | >100 |
| THB5 | Irregular | Umbonate | Lobate | Light yellow | 35.09 |
| THB6 | Circular | Raised | Entire | Yellow | 54.47 |
| THB17 | Circular | Convex | Entire | Dark yellow | 20.56 |
| THB18 | Circular | Flat | Entire | Light yellow | 55.76 |
| THB30 | Circular | Convex | Entire | Light white | 63.67 |
| THB31 | Irregular | Raised | Lobate | Waxy | 45.27 |
| THB32 | Circular | Convex | Entire | White | 65.13 |
| THB44 | Circular | Convex | Entire | Light yellow | >100 |
| THB45 | Circular | Raised | Entire | Transparent White | 87.89 |
| THB56 | Irregular | Raised | Undulate | Dull white | >100 |
| THB57 | Circular | Raised | Entire | Whitish yellow | >100 |
| THB58 | Irregular | Raised | Undulate | Light whitish yellow | >100 |
| THB69 | Circular | Flat | Entire | Waxy | 90.31 |
| THB70 | Irregular | Flat | Undulate | White | 77.72 |
| THB83 | Circular | Flat | Entire | Dull white | >100 |
| THB84 | Circular | Raised | Entire | Whitish yellow | 79.17 |
| THB85 | Irregular | Raised | Undulate | Waxy | 75.30 |
| THB97 | Circular | Raised | Entire | Whitish yellow | >100 |
| THB98 | Circular | Flat | Entire | Light yellow | >100 |
| THB99 | Circular | Raised | Entire | White | >100 |
| THB110 | Irregular | Flat | Undulate | Dull white | 68.39 |
| THB111 | Circular | Raised | Entire | White | >100 |
| THB112 | Irregular | Flat | Undulate | Dull white | 78.14 |
| THB125 | Circular | Convex | Entire | Transparent yellow | >100 |
| THB126 | Circular | Raised | Entire | Waxy | 89.62 |
| THB127 | Circular | Flat | Entire | Transparent waxy | 71.35 |
| THB136 | Circular | Flat | Entire | Light orange | >100 |
| THB137 | Circular | Raised | Entire | Light yellow | 63.47 |
| THB138 | Circular | Raised | Entire | Blackish green | >100 |
| THB147 | Circular | Convex | Entire | Milky white | 61.32 |
| THB148 | Circular | Convex | Entire | Yellow | 89.04 |
| Positive control | Chloroquine | | | | 19.59 |
| | Artemether | | | | 4.09 |

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

## 4. Discussion

Marine microorganisms have attracted increasing attention in the search for new pharmaceutical or agrochemical lead structures[12]. Many marine microorganisms are symbiotic with marine sponges and other invertebrates. Their secondary metabolites might contribute to protecting their hosts by chemically mediated defense mechanisms from dangers like predation. There are evidences that, symbiotic or associated marine microorganisms are the true sources of bioactive metabolites originally isolated from their hosts. Thus, the microorganisms associated with marine animals and plants are expected to be potential sources for new natural bioactive agents[13-18]. Earlier investigations reported that, the marine bacteria associated with marine organisms have many biological activities[19-22]. The spread of multidrug resistant *P. falciparum* has highlighted the urgent need to develop new antiplasmodial agents from marine bacteria associated with sponges.

The present study has collected 12 *S. carteri* samples throughout the year at different seasons and all samples have reported to harbour bacterial isolates due to the filter feeding nature of sponges containing large numbers of associated bacteria[23,24]. The maximum count of bacteria was isolated in the month of November 2007 ($34\times10^4$ CFU/g). Likewise, Muscholl-Silberborn *et al* isolated $9.5\times10^4$ CFU/g of bacteria from *Chondrosia reniformis* collected from Mediterranean sea[25]. The present study also observed that, the bacterial stains 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 findings states that, THB17 showed antiplasmodial $IC_{50}$ value of 20.56 μg/mL and this could be comparable to the positive control chloroquine. Many researchers found that, the marine microorganisms showed potential antiplasmodial activity against *P. falciparum*[26-28]. According to Rasoanaivo *et al*, 9%, 50% and 41% of extracts from isolated bacterial isolates were classified as active, weakly active and inactive respectively[10].

The biochemical constituent analysis of potential extracts showed the presence of reducing sugars and alkaloids. The mode of action could be due to the inhibition of *P. falciparum* merozoites invasion into the erythrocytes[29] and disruption of *P. falciparum* rosettes[30] by the carbohydrates; inhibition of *P. falciparum* fatty acid biosynthesis[31], inhibition of hemozoin biocrystallization by the alkaloids[32]. Otoguro *et al* reported that, polysaccharides, polyketides and polysaccharide derivatives are having potential antiplasmodial activity[33-37]. Stierle *et al* report supports the present study that, the presence of alkaloids and reducing sugars showed potential *in vitro* antiplasmodial activity[38]. Earlier investigations reported that a number of alkaloids from marine sources possess antimalarial activity[39,40]. Moreover, alkaloid derivatives *viz.*, 8-hydroxy-manzamine, manazamine, cycloprodigiosin, heptyl prodigiosin, ascosalipyrrolidinone A were reported from marine microbial community[41-43]. El Sayed *et al* reported that the marine sponge associated microbial alkaloids possess antiplasmodial activity[44]. These findings could encourage the microbes derived compounds for the antiplasmodial drug development.

It is concluded from the present study that the *S. carteri* associated bacterial isolates proved as massive source to come across the novel antiplasmodial drugs. 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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