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In vitro antiplasmodial activity of marine sponge *Clathria indica* associated bacteria against *Palsmodium falciparum*

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ABSTRACT

Objective: To identify the possible antiplasmodial drugs from bacteria associated with marine sponge *Clathria indica*. **Methods:** *Clathria indica* 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 μ g.mL^{-1}) from isolated bacterial isolates were screened for antiplasmodial activity against *Palsmodium falciparum* and potential extracts were also screened for biochemical constituents. **Results:** The count of bacterial strains were maximum in November 2007 (19×10^4 CFU.g^{-1}) and the average count was maximum during the monsoon season (107×10^3 CFU.g^{-1}). Thirty one morphologically different bacterial isolates were isolated from *Clathria indica* and the ethyl acetate bacterial extracts were screened for antiplasmodial activity against *Palsmodium falciparum*. The antiplasmodial activity of a isolate THB23 (IC_{50} 28.80 μ g.mL^{-1}) extract is highly comparable with the positive control chloroquine (IC_{50} 19.59 μ g.mL^{-1}) and 17 bacterial extracts which showed IC_{50} value of more than 100 μ g.mL^{-1}. 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 strains after 48 h of incubation. The *in vitro* antiplasmodial activity might be due to the presence of carbohydrates and alkaloids in the ethyl acetate extracts of bacterial isolates. **Conclusions:** The ethyl acetate extracts of THB23 possesses novel compounds for the development of antiplasmodial drugs.

1. Introduction

Marine microorganisms are emerging as an exciting resource for the discovery of novel bioactive compounds. Marine microorganisms exhibit a wide range of biological activity such as antibacterial, antifungal, anti-inflammatory, antitumor, Cytotoxic, algicidal, antiviral and immunosuppressive[1-12]. The microorganisms associated with marine animals produce pharmaceutically valuable secondary metabolites, since the marine microorganisms live in a significantly different environment from those of the terrestrial microorganisms. Sponges are well known to be hosts for a large community of microorganisms, such as bacteria and fungi and some of these microbes are probably host specific[13-27]. That exceeds the number of bacteria in seawater by two-three orders of magnitude[28]. Earlier investigations reported that, the microbial communities associated with sponges produce novel bioactive natural compounds with host specificity[29,30]. As marine sponges are a rich source of diverse bacterial populations, the present investigation was carried out to explore the antiplasmodial activity of *Clathria indica* associated bacteria from Palk Strait region.

2. Materials and methods

2.1. Isolation of sponge associated bacteria

Marine sponge *Clathria indica* was collected by by-catch at Thondi (Lat. 9° 44′ 11″ N and Lon. 79° 1′ 13″ 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

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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.

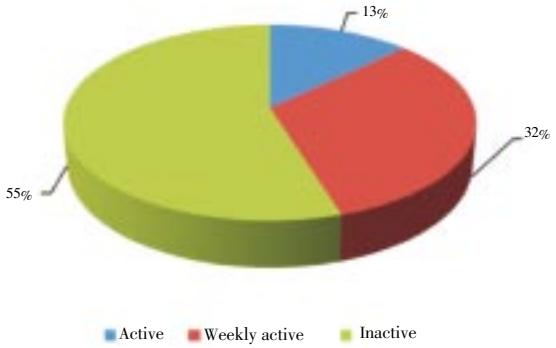

Figure 1. Percentage of antiplasmodial activity IC_{50} values of *Clathria indica* associated bacterial strains against *Palsmodium falciparum*.

2.2. Mass cultivation of total heterotrophic bacteria

A loopful inoculum of isolated bacterial strains 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 isolated bacterial stains were adjusted to pH 5.0 using 1N 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 *Palsmodium falciparum* obtained from the Jawaharlal Nehru Centre for Advanced Scientific Research, Indian Institute of Science, Bangalore, India. *Palsmodium falciparum* are cultivated in human O Rh^+ red blood cells using RPMI 1640 medium (HiMedia Laboratories Private Limited, Mumbai, India)[31] supplemented with O Rh^+ serum (10%), 5% sodium bicarbonate (HiMedia Laboratories Private Limited, Mumbai, India) and 40 μg.mL^{-1} of gentamycin sulphate (HiMedia Laboratories Private Limited, Mumbai, India). Hematocrits were adjusted at 5% and parasite cultures were used when they exhibited 2% parasitaemia[32].

2.5. In vitro antiplasmodial assay

Filter sterilized extracts (100, 50, 25, 12.5, 6.25 and 3.125 μg.mL^{-1}) from 31 bacterial isolates were incorporated into 96 well tissue culture plate containing 200 μL of *Palsmodium falciparum* culture with fresh red blood cells diluted to 2% hematocrit. Negative control was maintained with fresh red blood cells and 2% parasitized *Palsmodium falciparum* diluted to 2% hematocrit, positive control was maintained with parasitized blood cells culture treated with chloroquine and Artemether[33]. 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[34]. According to this norms, an extract is very active if $IC_{50} < 5$ μg.mL^{-1}, active 5 μg.mL^{-1} < IC_{50} < 50 μg.mL^{-1}, weakly active 50 μg.mL^{-1} < IC_{50} <100 μg.mL^{-1} and inactive $IC_{50} > 100$ μg.mL^{-1}.

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^{-1} 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[35].

Table 1
Counts of associated bacterial isolates from *Clathria indica*.

Sample No.	Month of Collection	THB × 10^4 CFU.g^{-1}	Season	THB× 10^3 CFU.g^{-1}
1	August 2007	3	Pre monsoon	27
2	September 2007	2		
3	October 2007	3		
4	November 2007	19	Monsoon	107
5	December 2007	11		
6	January 2008	2		
7	February 2008	4	Post monsoon	23
8	March 2008	2		
9	April 2008	1		
10	May 2008	2	Summer	53
11	June 2008	7		
12	July 2008	7		

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^{-1})
THB-10	Irregular	Raised	Filamentous	White	51.08
THB-11	Circular	Flat	Entire	Brownish yellow	58.83
THB-21	Irregular	Raised	Undulate	Waxy	35.47
THB-22	Irregular	Raised	Undulate	Pale	42.84
THB-23	Circular	Raised	Entire	Transparent yellow	28.80
THB-36	Circular	Raised	Entire	Waxy	75.30
THB-37	Irregular	Raised	Undulate	Light yellow	34.61
THB-49	Circular	Raised	Entire	Waxy	>100
THB-50	Circular	Convex	Entire	Transparent waxy	>100
THB-51	Circular	Raised	Entire	Transparent waxy	>100
THB-61	Circular	Raised	Entire	Centre white, surrounding transparent yellow	>100
THB-62	Circular	Raised	Entire	White	69.00
THB-74	Circular	Raised	Entire	Light yellow	>100
THB-75	Irregular	Raised	Undulate	White	>100
THB-76	Circular	Flat	Entire	Lemon yellow	56.41
THB-88	Irregular	Flat	Undulate	White	>100
THB-89	Circular	Raised	Entire	Light yellow	>100
THB-90	Circular	Convex	Entire	Dull white	>100
THB-102	Circular	Raised	Entire	Waxy	>100
THB-103	Circular	Flat	Entire	Orange	92.58
THB-104	Circular	Raised	Entire	Yellow	>100
THB-116	Irregular	Raised	Undulate	Light yellow	>100
THB-117	Circular	Raised	Entire	Yellow	>100
THB-118	Circular	Raised	Entire	Light brown	88.61
THB-130	Circular	Raised	Entire	Light yellow	>100
THB-131	Circular	Raised	Entire	Whitish yellow	>100
THB-141	Irregular	Flat	Curled	White	>100
THB-142	Circular	Convex	Entire	Orange	86.92
THB-151	Irregular	Raised	Undulate	Waxy	96.37
THB-152	Circular	Raised	Entire	Light yellow	>100
THB-153	Circular	Raised	Entire	Transparent white	74.32
Positive control					
Chloroquine					19.59
Artemether					4.09

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

3. Results

The counts of isolated bacterial isolates from the marine sponge *Clathria indica* samples are represented in Table 1. The bacterial count was maximum in the month of November 2007 (19×10^4 CFU.g^{-1}) and minimum in the month of April 2008 (1×10^4 CFU.g^{-1}). The average count was maximum during the monsoon season (November – January) (107×10^3

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

Biochemical Constituents	THB21	THB22	THB23	THB37
Reducing sugars	+	+	+	+
Amino acids	−	−	−	−
Proteins	−	+	−	−
Alkaloids	+	+	+	+
Steroids	−	−	−	−
Triterpenoids	−	−	−	−

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

CFU.g^{-1}) and followed by summer season (May − July) (53× 10^3 CFU.g^{-1}). A total of 31 different bacterial isolates were isolated from *Clathria indica* based on the morphological characteristics (Table 2). The extract of THB23 (28.80 μg.mL^{-1}) showed minimum level of IC_{50} value and followed by THB37 (34.61 μg.mL^{-1}), THB21 (35.47 μg.mL^{-1}) and THB22 (42.84 μg.mL^{-1}). The extracts from THB10, THB11, THB36, THB62, THB76, THB103, THB118, THB142, THB151 and THB153 showed IC_{50} values between 50 to 100 μg.mL^{-1}. Among 31 bacterial extracts screened for antiplasmodial activity, THB49, THB50, THB51, THB61, THB74, THB75, THB88, THB89, THB90, THB102, THB104, THB116, THB117, THB130, THB131, THB141 and THB152 showed IC_{50} values more than 100 μg.mL^{-1} (Table 2). The screened extracts showed significant antiplasmodial activity (P<0.05) between the concentrations and time of exposure.

The microscopic observation of uninfected erythrocytes added with the ethyl acetate extracts from 31 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 bacterial isolates have variety of biochemical constituents, namely alkaloids and reducing sugars (Table 3).

4. Discussion

Malaria is a devastating infectious disease and a major cause of morbidity and mortality throughout the world. There are up to 500 million clinical cases and 3 million deaths annually[36]. The drugs currently used to treat malaria come from three families such as quinolines, antifolates and artemisinin derivatives[37]. This major public health problem is aggravated by the widespread diffusion of drug resistant strains of *Palsmodium falciparum*. This increasing resistance to multiple drugs may be the result of defective DNA repair or other pathways responsible for genomic stability[38]. Antiplasmodial compounds from natural resources would be the alternative to overcome the resistance problem. In that, the marine invertebrates play a major role and harbor numerous microorganisms within their tissues[14]. Sponge mesohyl was referred as "micro-environments" providing a broad variety of ecological niches[39].

All the collected *Clathria indica* sponge samples reported to harbour associated bacteria and this might be due to bacteria can contribute up to 40% of the sponge biomass and are probably permanently associated with the host sponge unless they are disturbed by external stress factors[40-44]. The present study also observed variable counts in different months and different seasons. Similarly, Taylor et al. reported that, the substantial variability of microbial communities between different species of sponges although there was little variability of microbial communities within each sponge species[45]. Taylor et al. observed that, the sponge associated microorganisms considerably varied with geographic[14].

In vitro screening for antiplasmodial activity is one of the key tools for identification lead compounds for new antiplasmodials. In this study, the THB23 showed excellent antiplasmodial activity and it could be comparable with the positive control chloroquine. The antiplasmodial nature of sponge associated microbes due to the microbes can sense, adapt and respond to their environment quickly and can compete for defense and survival by the generation of unique secondary metabolites. The marine microorganisms showed potential antiplasmodial activity against *Palsmodium falciparum*[46-48]. According to Rasoanaivo *et al*. 13%, 32% and 55% of extracts from isolated bacterial isolates were classified as active, weakly active and inactive respectively (Figure 1)[34].

The ethyl acetate extracts of potential strains showed the presence of reducing sugars and alkaloids. The presence of these biochemical constituents in the potential isolates extracts, especially sugars and alkaloids[49,50]. The mode of action could be due to the inhibition of *Palsmodium falciparum* merozoites invasion into the erythrocytes[51] and disruption of *Palsmodium falciparum* rosettes[52] by the carbohydrates; inhibition of *Palsmodium falciparum* fatty acid biosynthesis[53], inhibition of hemozoin biocrystallization by the alkaloids[54]. Alkaloids are nitrogenous compounds derived from many biogenetic precursors and possessing antimalarial activity[55]. El Sayed et al. reported that, the marine sponge associated microbial alkaloids (curcuphenol) have antiplasmodial activity[56]. Numerous alkaloids isolated from marine sources have antimalarial activity[57]. These findings could encourage the sponge associated bacteria derived compounds for the

antiplasmodial drug development. It is concluded that, the ethyl acetate extract of *Clathria indica* associated bacterial isolate THB23 proved as a massive resource to develop potential antiplasmodial drugs. This justifies our continuing research to fractionate the *Clathria indica* associated bacterial extracts to determine the active principles responsible for antiplasmodial activity.

Conflict of interest statement

We declare that we have no conflict of interest.

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