Evaluation of the potential of the marine sponges of the Zanzibar Island to yield antimalarial and antimicrobial active compounds

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Abstract: Emergence of new and re-emergence of old infections continue to elude prospects of reducing morbidity and mortality caused by microbial infections. Trends of resistance to currently in use antimicrobials and antimalarials threaten to increase mortality caused by these infections. This study explores the potential of marine invertebrates as a source for new antimicrobials and antimalarials. The lactate dehydrogenase method was used to assay marine sponges for activity against *Plasmodium falciparum*, while the disc diffusion method was used to assay the extracts for antibacterial and antifungal activity. Extracts of some marine sponges from the Zanzibar Island exhibited both antiplasmodial and antimicrobial activities. Among the 55 marine sponge extracts that were tested 23 (41.8%) inhibited *Plasmodium falciparum* W2 strain by more than 50% at both 250 and 50 μg/ml concentrations. Moderate polar extracts were more active against *Plasmodium falciparum* W2 strain than polar and non-polar extracts. None of the 12 extracts that were tested on *Plasmodium falciparum* strain D6 exhibited inhibitory activity reaching 50%. Among 18 marine sponge extracts that were tested for antimicrobial activity 12 (66.7%) showed activity against one or more of the bacteria and fungi used ranging from weak to strong on an arbitrary criterion. The ethyl acetate extracts of *Agelas mauritania* and *Oceanopia* sp. exhibited high activity against the fungi *Candida albicans* and *Cryptococcus neoformans***.** The best antibacterial profile was exhibited by ethyl acetate extracts of *Aplysinopsis* sp., *Halichondrida* sp. 1 and *Oceanopia* sp. In conclusion, these results support the need for intensified efforts to search for active antimalarial and antimicrobial compounds from the Zanzibar marine sponges.

Keywords: Antimalarial, antimicrobial activity; marine sponges, Zanzibar

Introduction

The optimism of the 1950s and 1960s of a world without infections is gradually being replaced by an era of pessimism characterized by widespread emergence of resistance among most of the major pathogens (Raghunath, 2008; Andersson & Hughes, 2010). Microbial infections are still the major cause of mortality the world over (World Health Report, 2003). While the HIV/AIDS pandemic as well as emergent and re-emergent infections have brought a new dimension to antimicrobial chemotherapy (Nordberg *et al*., 2004), the steady discovery of novel antibiotics in the period 1940-1980 has not been sustained, and as a result the 1990s saw only one new antibiotic class, the oxazolidinones joining the approval list (Raghunath, 2008) .

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Like bacterial infections, malaria chemotherapy is challenged by the emergence of drug resistant *Plasmodium falciparum* (Hyde *et al*., 2005). Widespread *P. falciparum* resistance to the most affordable agents, particularly chloroquine and sulfadoxine–pyrimethamine, undermine recent achievements made in malaria chemotherapy (Fegan *et al*., 2007; Hyde *et al*., 2005). Recently, artemisinin-based combination therapies (ACTs) were recommended by the World Health Organization (WHO) Roll Back Malaria group for treatment of uncomplicated malaria. ACTs are clinically effective, and may reduce malaria transmission rates and the potential for resistance development (Achuyt *et al*., 2007; Bousema *et al*., 2006; Sutherland *et al*., 2005). However, ACTs face great pharmacological challenges with regard to variable drug bioavailability, drug interactions, and the long half life of partner drugs that have implication in the development of resistant parasite mutants (German & Aweeka, 2008; Kremsner & Krishna, 2004; Talisuna *et al*., 2004). Recent reports suggest that there may already be signs of the emergence of *invitro* resistance to some artemisinins (Jambou *et al*., 2005; Uhlemann *et al*., 2005). Reports of rampant resistance to antimalarial and antimicrobial agents, is a loud trumpet calling for intensified efforts to search for new chemotherapeutic agents.

This study, led by promising results of drug bio-prospecting among marine invertebrates such as sponges, tunicates, bryozoans, and molluscs (Abdelmohsen *et al*., 2010; Orhan *et al*., 2010; Arai *et al*., 2009; Blunt *et al*., 2006; Haefner, 2003) seeks to further initiatives to discover antimicrobial and antimalarial drugs by evaluating the potential of some marine sponges collected off the Zanzibar coasts.

Materials and methods

Collection of sponges

Sponges were collected by snorkelling and SCUBA diving in diverse habitats (e.g. shallow and deep coral reefs, mangrove channels, sea grass habitats, lagoons, and estuaries) located on different parts of Zanzibar island shores. About 300-500 g pieces of sponges were collected and put into zip lock bags underwater to avoid mixing them. Prior to collection underwater photographs were taken for future identification. From each species a voucher sample was taken for taxonomical morphological identification. All samples were preserved at low temperature (-20^{o}C) in the laboratory.

Extraction and creation of fraction libraries

Freeze dried or wet samples were soaked twice in methanol and twice in a mixture of equal volumes of methanol/dichloromethane (1:1). Each soaking lasted 24 h. After filtration solvents were evaporated under reduced pressure in a rotary evaporator and the extracts were combined. Aqueous combined extracts were then consecutively extracted with hexane, dichloromethane and n-butanol to obtain extracts with varying polarities.

Screening for antiplasmodial activity

Animalarial activity was assayed using plasmodium lactate dehydrogenase (pLDH) (Kaddouri *et al*., 2006). Chloroquine resistant *Plasmodium falciparum* strains W2 and D6 were used. Cultures of *P. falciparum* were maintained *in vitro* in human blood cells (O+ve) diluted to 5% hematocrit with RPMI 1640 medium (10% human O+ serum). Each extract was dissolved in DMSO and diluted with RPMI 1640 medium before testing. The concentration of DMSO in the test never exceeded 0.1%. Portions (50 µl each) of diluted extract were

dispensed into 96-well microtiter trays so as to yield final test concentrations of 250 and 50 μg ml^{-1}. All tests were performed in duplicate. To each well was added 50 μl of human erythrocytes (O+ve, diluted to 5% hematocrit) with 1% parasitemia (dilutions to 1% parasitemia were made with uninfected washed erythrocytes). Two series of controls were performed, one with parasitized blood without extracts and another with uninfected erythrocytes without extracts. Incubation was carried out in a 5% O_2-5% CO_2-90% N_2 gas phase for 72 h at 37°C. After incubation 100 μl of Malstat was added to each well. This was followed by addition of NTB/PES mixture (1:1). The plates were then de-bubbled, covered with aluminium foil and kept in a dark place for 1-2 h. Absorbance (optical density, OD) was measured at 260 nm. Percent inhibition for the two concentrations was calculated.

Antimicrobial test

Antibacterial and antifungal activities were tested by the disc diffusion method (Singh *et al*., 2002). Bacteria and fungi used include: *Staphylococcus aureus* (NCTC 6571), Pseudomonas *aeruginosa* (NCTC 10662), *Bacillus anthracis* (NCTC 10073), *Proteus mirabilis* (NCTC 10975), *Shigella dysenteriae* (clinical isolate), and two fungi, *Candida albicans* (Strain HG 392), and *Cryptococcus neoformans* (clinical isolate) were used. Filter paper discs (Whatman no.1; 6 mm diameter) were impregnated with crude extracts (5 mg/disc) or standard drugs (10 μg/disc gentamycin for bacteria, and 20 μg/disc clotrimazole for fungi). The discs were placed on Mueller Hinton agar plates (for bacteria) and Saborauld's dextrose agar plates (for fungi) and incubated at 37° C, for 24 h. Each test was done in triplicate. Controls were blank discs impregnated with solvent. Inhibition zones were calculated as the difference between disc diameter (6 mm) and the diameters of inhibition (Hewitt & Vincent, 1989). Activity index (AI) was calculated as the ratio of the mean inhibition zone (IZ) for the sample to that for standard drug (Singh et al., 2002).

Results

Antimalarial tests

The results of antimalarial activity presented in Table 1 show that 23 out of the 55 extracts tested (41.8%) inhibited *Plasmodium falciparum* W2 strain by more than 50% at both 250 and 50 μg/ml concentrations. Seventeen out of these extracts displayed high potency at both low and high concentrations; these include extracts in Table 1 with entries 3, 10, 11, 13, 14, 15, 19, 23, and 27. Others are entries 30, 36, 39, 41, 44, 45, 47, and 52. The other extract entries exhibiting activity at 50% or higher are Table 1 entries number 12, 26, 33, 40 and 46. None of the 12 extracts that were tested on *Plasmodium falciparum* strain D6 exhibited inhibitory activity reaching 50% (Table 2).

Table 1: *Invitro* antiplasmodial activity of marine sponge extracts against *Plasmodium falciparum* Strain W2

Entry	Sponge Code	Sponge ID	Extract type	Mean % Inhibition at 250μg/ml	Mean % Inhibition at 50 μg/ml
1	Z04A56	*Agelas mauritania*	EA	81.9	26.4
2	ZO4A 157	*Ancorinidae sp.*	EA	29.4	32.2
3	Z04A114	*Aplysinopsis sp.*	EA	95.9	88.1
4	Z04A94	*Axinellidae sp.*	ME	92.2	1.96
5		*Axinellidae sp.*	DM	50.5	2.0
6	Z04A65	*Callyspongia sp.*	ME	64.7	17.9
7	Z04A20	*Chalinidae sp. 1*	EA	81.6	64.6
8	Z04A165	*Chalinidae sp. 2*	EA	85.7	40.9
9	ZO4A 83	*Clathria sp.*	BU	37.3	36.4
10	Z04A149	*Cliona sp. 2*	EA	88.8	83.3
11	Z04A158	*Cliona sp.1*	EA	82.9	87.1
12	Z04A21	*Cribrochalina sp.*	DM	71.3	53.4
13		*Cribrochalina sp.*	ME	100	84.1
14	Z04A101	*Halichondrida sp.1*	EA	94.8	87.3
15	Z04A111	*Halichondrida sp.2*	DM	64.8	70.5
16	ZO4A 49	*Halichondrida sp.3*	EA	31.4	33.6
17	Z04A130	*Halichondridae sp. 1*	HX	70.4	8.2
18	Z04A151	*Halichondridae sp.2*	HX	56.9	26.7
19		*Halichondridae sp.2*	DM	90.7	85.3
20	ZO4A 162	*Halisarca sp.*	EA	36.2	39.2
21	Z04A109	*Haplosclerina sp.*	EA	69.8	18.5
22	ZO4A 119	*Haplosclerina sp. 2*	EA	40.6	33.3
23	Z04A50	*Haplosclerina sp.1*	DM	88.1	61.2
24		*Haplosclerina sp.1*	HX	0	0
25	ZO4A 36	*Haplosclerina sp.3*	BU	34	27.6
26	Z04A150	*Hymeniacidon sp.*	EA	67.5	63.8
27	Z04A45	*Jaspis sp.*	DM	95.0	86.6
28	Z04A45	*Jaspis sp.*	BU	42.4	0
29	Z04A45	*Jaspis sp.*	HX	52.7	23.9
30	Z04A80	*Liosina sp.*	DM	72.4	70.9
31		*Liosina sp.*	HX	63.2	0
32	Z04A132	*Myxillina sp.1*	EA	94.4	37.5
33	Z04A105	*Myxillina sp.2*	HX	78.9	50.8
34	Z04A67	*Niphatidae sp. 1*	DM	52.4	6.4
35	ZO4A 133	*Niphatidae sp.2*	EA	34.2	31.2
36	Z04A120	*Oceanopia sp.*	EA	96.7	91.2
37	Z04A192	*Petrosiidae sp.*	EA	25.4	0
38	Z04A74	*Phloeodictyidae sp.*	EA	59.2	34.2
39	Z04A24	*Pseudoceractina Arabica*	ME	80.6	80.2
40		*Pseudoceractina Arabica*	DM	61.6	56.4
41	Z04A156	*Pseudoceractina clavata*	EA	95.9	82.7
42	Z04A147	*Spirastrella sp.*	HX	59.9	9.5
43	Z04A26	*Spongidae*	ME	93.0	44.8
44	Z04A143	*Stylisa carteri*	EA	88.2	94.2
45	Z04A145	*Suberites sp.*	EA	94.4	78.9
46	Z04A155	*Tedania sp. 1*	HX	65.7	54.4
47	Z04A152	*Tedania sp. 2*	EA	91.9	94.4
48	Z04A17	*Tedaniidae sp.*	EA	63.1	35.1
49	Z04A07	*Tetillidae sp.*	EA	26.2	0
50		*Tetillidae sp.*	HX	29.7	22.4
51		*Tetillidae sp.*	DM	33.5	34.5

52	Z04A81	*Theonella swinhoei*	EA	97.3	96.3
53	ZO4A 95	*Thorectidae sp.*	EA	39.9	43.5
54	ZO4A 53	*Verongida sp.*	BU	42.6	40.9
55		*Verongida sp.*	HX	39.9	29.4

Key: BU: n-butanol (n-BuOH) extract; DM: dichloromethane (CH_2Cl_2) extract; EA: ethylacetate (EtOAc) extract; HX: n-hexane (n-C_6H_{14}) extract; ME: methanol (MeOH) extract

Antimicrobial tests

The results in Table 3 show that among 18 marine sponge extracts that were tested for antimicrobial activity 12 (66.7%) showed activity against one or more of the organisms used ranging from weak to strong on the following arbitrary criterion: 0-5 mm of inhibition = very weak activity; 6-10 mm of inhibition = weak activity; 11- 20 mm of inhibition = good activity; >20 mm inhibition = strong activity. Six extracts exhibited weak antibacterial activity against one or more organism. Two extracts exhibited good activity against *Candida albicans*; these are ethyl acetate extracts of *Halichondrida* sp. 1 and *Oceanopia* sp. The ethyl acetate extracts of *Agelas mauritania, Hymeneciadon* sp., *Oceanopia* sp., and hexane extract of *Tedania* sp. 1 exhibited good to strong activity against *Cryptococcus neoformans* (Table 3). *Aplysinopsis* sp. ethyl acetate extract showed good activity against both *Bacillus anthracis* and *Staphylococcus aureus*; *Halichondria* sp. 1 ethyl acetate extract was active against *Bacillus anthracis* (strong activity), *Proteus mirabilis* (good activity), and *Staphylococcus aureus* (good activity). The ethyl acetate extract of *Oceanopia* sp. also exhibited good antibacterial activity against *Shigella dysentriae, Bacillus anthracis, Proteus mirabilis*, and *Staphylococcus aureus*.

Table 2: Results of *in vitro* antiplasmodial activity testing of sponge extracts against *Plasmodium falciparum* Strain D6

Entry	Sponge ID	Extract type	Mean % Inhibition at 250μg/ml	Mean % Inhibition at 50 μg/ml
1	*Chalinidae* sp.2	EA	26.7	9.3
2	*Clathria* sp.	DM	23.6	20.8
3	*Clathria* sp.	HX	35.1	28.6
4	*Haplosclerida* sp.	EA	23.1	22.5
5	*Haplosclerina* sp.3	DM	22.9	26.3
6	*Haplosclerina* sp.3	HX	28.7	25.9
7	*Haplosclerina* sp.4	HX	33	17.4
8	*Haplosclerina* sp.4	DM	29.9	11.5
9	*Haplosclerina* sp.5	EA	13.7	11.5
10	*Spirastrella* sp.	BU	9.9	11.7
11	*Tedaniidae* sp.	EA	28.3	22.8
12	*Verongida* sp.	DM	29.0	25.9

Key: BU: n-butanol (n-BuOH) extract; DM: dichloromethane (CH_2Cl_2) extract; EA: ethylacetate (EtOAc) extract; HX: n-hexane (n-C_6H_{14}) extract; ME: methanol (MeOH) extract

Among the 18 extracts tested 6 (33.3%) had neither antibacterial nor antifungal activity (Table 3). These include ethyl acetate extracts of *Cliona* sp. 1 and sp. 2, ethyl acetate extract of *Halichondrida* sp. 4 and sp. 1, hexane extract of *Myxillina* sp. 2, and ethyl acetate extracts of *Pseudoceratina clavata* and *Suberites* sp. 1.

The results of the inhibition of bacterial and fungal growth by sponges extracts are summarised in Table 3. Results are reported as inhibition zones (IZ; mm) with the corresponding activity index (AI). IZ are presented as mean ± SD (n =3). Inhibition zones exclude the disc diameter (6 mm); AI = IZ of test sample divided by IZ of standard drug.

Table 3: Inhibition of bacterial and fungal growth by sponges extracts (5mg/disc)

Sponge ID	*Extracts /drugs*	*S.dysentriae*		*B.anthracis*		*P.mirabilis*		*P.aeruginosa*		*S.aureus*		*C.albicans*		*C.neoformans*	
		IZ	AI	IZ	AI	IZ	AI	IZ	AI	IZ	AI	IZ	AI	IZ	AI
	Gent	21.0±0.5	1.0	23.0±0.7	1.0	23.0±0.5	1.0	18.0±0.3	1.00	25.0±0.7	1.00	NT		NT	0
	Clot	NT		NT		NT		NT		NT		20±0.5	1.00	18±0.5	1.0
Agelas Mauritania	EA	0	0	0	0	0	0	0	0	0	0	0	0	20.0±0.3	1.1
Aplysinopsis sp.	EA	0	0	15.3±0.2	0.7	6.0±0.1	0.3	0	0	13.0±0.3	0.5	0	0	0	0
Axinellidae sp.	ME	0	0	0	0	0	0	0	0	7.6±0.2	0.3	0	0	0	0
Cliona sp.1	EA	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Cliona sp.2.	EA	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Halichondrida sp.1	EA	7.0±0.2	0.3	22.3±0.1	1.0	18.3±0.1	0.8	7.0±0.5	0.39	17.3±0.7	0.7	15.3±0.4	0.8	0	0
Halichondrida sp.4	EA	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Halichondridae sp.2	DM	0	0	7.0±0.2	0.3	9.0±0.5	0.4	0	0	6.0±0.5	0.2	0	0	0	0
Haplosclerina sp.1	HX	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Hymeneciadon sp.	EA	8.6±0.6	0.4	9.6±0.5	0.4	0	0	0	0	0	0	0	0	19.6±0.4	1.1
Jaspis sp.	BU	0	0	8.3±0.3	0.4	10.3±0.5	0.4	0	0	10.0±0.5	0.4	0	0	0	0
Jaspis sp.	DM	0	0	6.0±0.3	0.3	0	0	0	0	0	0	0	0	0	0
Myxillina sp.2	HX	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Oceanopia sp.	EA	15.0±0.3	0.7	17.0±0.4	0.7	14.0±0.4	0.6	0	0	10.3±0.5	0.4	16.6±0.2	0.8	20.3±0.6	1.1
Pseudoceractina clavata	EA	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Stylisa carteri	EA	0	0	8.3±0.2	0.4	6.0±0.5	0.3	6.3±0.4	0.35	9.0±0.3	0.4	6.0±0.3	0.3	0	0
Suberites sp.	EA	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Tedania sp.1	HX	0	0	6.3±0.3	0.3	0	0	0	0	0	0	0	0	16.6±0.3	0.9

Key: BU: n-butanol (n-BuOH) extract; DM: dichloromethane (CH_2Cl_2) extract; EA: ethylacetate (EtOAc) extract; HX: n-hexane (n-C_6H_{14}) extract; ME: methanol (MeOH) extract; *Sdys: S. dysenteriae; Banth: Bacillus anthracis; Paer: Pseudomonus aeruginosa; Pmir P. mirabilis; Saur: Staphylococcus aureus; Calb: Candida albicans; Cneof: Candida neoformans;* Gent: gentamycin; Clot: clotrimazole.

Discussion

In general the moderate polar extracts (ethyl acetate and dichloromethane) were more active than the polar (methanol and butanol) and non-polar (hexane) extracts. All tested samples were inactive against *Plasmodium falciparum* D6 strains as shown in Table 2. The observed antiplasmodial activity in the marine invertebrate extracts is not unique as there are similar previous reports of isolation of antimalarial compounds from marine sources (Orhan *et al*., 2010; Lazaro *et a*l., 2002; El-Sayed *et al*., 2001). *Invitro* antiplasmodial activity of heptyl prodigiosin, isolated from a marine tunicate obtained from Philippines against *P. falciparum* 3D7 strain was reported to be similar to that of chloroquine [IC_{50} = 0.07 vs. 0.015 μM, respectively] (Lazaro *et al*., 2002). A detailed review on antimalarial compounds isolated from marine organisms is given by Mayer & Hamann (2005).

Despite the unfortunate situation that there were no enough extracts to determine the IC_{50} values for the active extracts, this study has been the first to report on antimalarial activity of marine invertebrates from the Zanzibar Islands. This should create more interest to build on the results and eventually isolate active antiplasmodial compounds from Tanzanian marine sponges.

More than 70 % of the species tested inhibited the growth of one or more of the micro-organisms tested. This wide distribution of antimicrobial activity is similar to that found in species from tropical and temperate marine waters (Rinehart *et al*., 1981). It is interesting to note that the ethyl acetate extracts of *Hymeniacidon sp*, *Agelas mauritania*, *Halichondrida sp.1*and *Oceanopia sp.*and hexane extract of *Tedania* sp. 1 possess very potent activities against the fungi *Candida albicans* and *C.neoformans.* Activities exhibited by 5 mg/ml extracts of these sponges are comparable to that of the azole antifungal clotrimazole. The ethyl acetate extract of *Agelas mauritania* showed selective activity against *C.neoformans*. It will thus be worthwhile to isolate the active antifungal compound/s from this extract. The antibacterial activities of *Aplysinopsis* sp., *Halichondrida* sp. 1, and *Oceanopia* sp. ethyl acetate extracts are also interesting enough, thus needing further work to isolate the active compounds. These extracts exhibited activity against both Gram positive and Gram negative bacteria. Antimicrobial properties displayed by the sponges tested in this study are comparable to results obtained on soft corals collected from the same coasts (Nyanda, 1992).
In conclusion, the results indicate potential to isolate active antiplasmodial, antibacterial and antifungal compounds from Tanzanian marine invertebrates.

Acknowledgements

We would like to thank all the technical staff of the Institute of Marine Sciences in Zanzibar for the great work of collecting the sponges. We also thank all the staff of the Department of Biological and Pre-clinical Studies at ITM for supportive work. This work was supported by a research grant from WHO/TDR.

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