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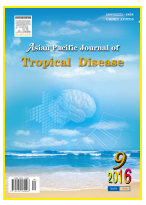

Microbiological research doi: 10.1016/S2222-1808(16)61119-2

In vitro antibacterial and antifungal activities of twelve sponges collected from the Anambas Islands, Indonesia

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ARTICLE INFO

Article history:
Received 19 Jul 2016
Received in revised form 3 Aug, 2nd revised form 10 Aug 2016
Accepted 15 Aug 2016
Available online 17 Aug 2016

Keywords:
Sponges
Antibacterial
Antifungal
Anambas Islands

ABSTRACT

Objective: To evaluate antimicrobial activities in methanolic extracts of twelve sponges collected from the Anambas Islands, Indonesia.
Methods: The antibacterial activity of methanolic extracts was tested against two Gram-positive bacteria, *viz. Bacillus subtilis* (ATCC 6633) and *Staphylococcus aureus* (ATCC 25923), and two Gram-negative bacteria, *viz. Eschericia coli* (ATCC 25922) and *Vibrio anguillarum* (ATCC 19264) using the disk diffusion assay. The antifungal activity was similarly tested against *Candida albicans* (ATCC 10231) and *Aspergillus niger* (ATCC 16404). The minimum inhibitory concentrations of promising sponges extracts were determined by the microdilution technique.
Results: All the sponge species in this study showed antimicrobial activities against at least one of the test strains. Antibacterial activities were observed in 66.7% of the sponges extracts, while 30.0% of the extracts exhibited antifungal activities. Among them, the extracts of the sponges *Stylissa massa* and *Axinyssa* sp. were the most active against four tested bacteria and the yeast *Candida albicans*. The sponge *Theonella swinhoei* and two species of *Xestospongia* also displayed significant activities against two fungal pathogens *Candida albicans* and *Aspergillus niger*.
Conclusions: Antimicrobial activities were demonstrated in extracts from various marine sponges collected from the Anambas Islands, Indonesia. The most promising sponges among them were *Stylissa massa* and *Axinyssa* sp. This is the first report of antimicrobial activity in extracts of marine sponges from the Indonesian Anambas Islands.

1. Introduction

Antibiotic resistance in pathogenic microorganisms is one of the global challenges of medical sciences in the 21st century. It has resulted in increased mortality and morbidity among the infected, adding enormously to the cost of healthcare. Recent strategies to address the problem include the search for new antibiotics from previously less explored sources such as oceans. Marine organisms, such as terrestrial species, are known to produce a wide variety of secondary metabolites to protect against pathogens, and some of these organisms could harbor various classes of antimicrobial substances[1].

Sponges have famously yielded the largest number of new metabolites of bio-medical significance as compared with any other plant or animal phylum from the marine environment[2]. To date, sponges account for some 4 850 compounds, or nearly 30% of all marine natural products discovered so far[1]. The chemical compounds isolated from marine sponges have a variety of pharmacological activities, such as anticancer, anti-infective, anti-inflammatory, neuroprotective, antifouling and other bioactivities[3,4].

Indonesian coastal waters support a wide diversity of marine life. Marine invertebrates, in particular, are rich in natural products. They are hence frequent targets in the continuing search for bioactive metabolites[5]. As reported in more than 70 publications, Indonesian marine macroorganisms such as sponges,

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Foundation Project: Supported by the Coral Reef Rehabilitation and Management Program – Coral Triangle Initiative (Grant No. COREMAP CTI-LIPI 2016 No. 10876401/ADB LOAN No. 3094-INO).
The journal implements double-blind peer review practiced by specially invited international editorial board members.

algae, ascidians and soft corals have been the source of more than 100 bioactive compounds[6]. In the course of this research program, twelve marine sponges collected from the Anambas Islands were analyzed. Here, the methanolic extracts of these sponges for antimicrobial activities were screening against four human pathogenic bacteria and two pathogenic fungi. The most active extracts will be studied further with the view to identify and purify the antimicrobial compounds.

2. Materials and methods

2.1. Sponge samples

Twelve marine sponges were collected from six sites of the Anambas Islands by scuba diving. The sponge species, locations, and depths of collection were listed in Table 1. Identification of the sponges was based on their morphological characteristics. A voucher record of each specimen was deposited at the Research Center for Oceanography of Indonesian Institute of Sciences. The collected sponges were immediately frozen at –20 ℃ prior to extraction.

2.2. Extractions

Each sponge sample with 200 g wet weight was thawed, and then extracted with 500 mL methanol at room temperature for 24 h. The sample was filtered and the residue was re-extracted with methanol (3 × 500 mL). Then, each extract of sponges was evaporated at reduced pressure to obtain crude methanolic extracts that were stored in a deep freezer until used. Each extract of sponges was examined for antibacterial and antifungal activites.

2.3. Microbial strains

Six reference strains of human pathogens were used in this research, including two Gram-negative bacteria *Eschericia coli* (ATCC 25922) (*E. coli*) and *Vibrio anguillarum* (ATCC 19264) (*V. anguillarum*), two Gram-positive bacteria *Staphylococcus aureus* (ATCC 25923) (*S. aureus*) and *Bacillus subtilis* (ATCC 6633) (*B. subtilis*), and two fungal strains *Aspergillus niger* (ATCC 16404) (*A. niger*) and *Candida albicans* (ATCC 10231) (*C. albicans*).

2.4. Antimicrobial assays

2.4.1. Agar diffusion test

Screening of antibacterial and antifungal activites in the sponge crude extracts was carried out using agar disk diffusion method as described by Qaralleh *et al.*[7] with slight modifications. Concisely, inoculums containing 10^7 CFU/mL were spread on Mueller-Hinton agar plates for antibacterial activity, while 10^4 CFU/mL were spread on potato dextrose agar for antifungal activity. Sterile forceps, sterile filter paper disks (6 mm diameter) containing crude extracts (5 mg) and standard antibiotics used for bacterial infections, chloramphenicol (30 mg) or 100 mg amphotericin B for fungal infections and methanol as solvent control were laid on the agar surface. The plates were incubated at 37 ℃ for 24 h for the bacterial inoculums and at room temperature (18–20 ℃) for 24–48 h for the fungal inoculums. The antibacterial and antifungal activities were evaluated by measuring the zone of growth inhibition surrounding the disks. All the assays were performed in triplicate.

2.4.2. Microdilution method

Microdilution method was used to evaluate the minimum inhibitory concentration (MIC) of the sponges extracts, which showed good activity (growth inhibition halos more than 9 mm in the disk diffusion assay) as described by Qaralleh *et al.*[7]. Tests were performed in 96-well round bottom sterile culture plates using the Infinite® 200 PRO microplate reader (Tecan Austria GmbH, Grödig, Austria). The assay plates were filled with Mueller-Hinton broth medium in serial dilutions ranging from 100 to 0.78 μg/mL of *S. massa* and *Axinyssa* sp. extracts, chloramphenicol or methanol and the test microorganism (10^7 CFU/mL). The turbidity in each well was measured at 600 nm, after 24 h of incubation

Table 1
Sponge species and places of collection.

Sponges	Places of collection	Depth (m)	Voucher specimen number
Dasychalina fragilis (*D. fragilis*)	Pulau Batu Belah (3°12'31.89" N, 106°17'42.16" E)	1–5	1ANBL2015
Stylissa massa (*S. massa*)	Pulau Batu Belah (3°12'31.89" N, 106°17'42.16" E)	1–5	2ANBL2015
Theonella swinhoei (*T. swinhoei*)	Pulau Gosong (3°12'27.58" N, 106°19'14.44" E)	5–10	3ANBL2015
Xestospongia sp. 1	Pulau Gosong (3°12'27.58" N, 106°19'14.44" E)	5–10	4ANBL2015
Pericharax heteroraphis (*P. heteroraphis*)	Pulau Mandarin (3°16'42.96" N, 106°24'14.35" E)	5–10	5ANBL2015
Axinyssa sp.	Pulau Mandarin (3°16'42.96" N, 106°24'14.35" E)	1–5	6ANBL2015
Thrinacophora cervicornis (*T. cervicornis*)	Pulau Samak (3°14'52.77" N, 106°26'35.56" E)	1–5	7ANBL2015
Haliclona fascigera (*H. fascigera*)	Pulau Samak (3°14'52.77" N, 106°26'35.56" E)	1–5	8ANBL2015
Callyspongia sp.	Pulau Penggending (3°9'17.65" N, 106°23'39.22" E)	1–5	9ANBL2015
Hyrtios erectus (*H. erectus*)	Pulau Penggending (3°9'17.65" N, 106°23'39.22" E)	1–5	10ANBL2015
Xestospongia sp. 2	Pulau Penjaul (3°8'34.25" N, 106°22'49.53" E)	5–10	11ANBL2015
Cinachyrella sp.	Pulau Penjaul (3°8'34.25" N, 106°22'49.53" E)	5–10	12ANBL2015

periods at 37 ℃.

MIC for fungal strains was carried out using 96-well plate. Each well contained potato dextrose broth, serial dilutions of *T. swinhoei*, *Xestospongia* sp. 1, *Callyspongia* sp. and *Xestospongia* sp. 2 extracts, amphotericin B or methanol and the test yeast strains (10^4 CFU/mL). The yeast growth was measured at 494 nm using the Infinite® 200 PRO microplate reader, after 48 h incubation periods at room temperature (18–20 ℃).

3. Results

The identification of sponge species and their sampling sites were summarized in Table 1. The antimicrobial activities of the twelve sponges extracts were summarized in Table 2.

Table 2
Antimicrobial activities in methanolic extracts of twelve marine sponges.

Sponge species	Zone of inhibition (mm)					
	S. aureus	*B. subtilis*	*E. coli*	*V. anguillarum*	*C. albicans*	*A. niger*
D. fragilis	7.5	7.4	0.0	6.5	3.2	1.3
S. massa	18.1	14.3	11.8	12.3	9.7	7.1
T. swinhoei	10.7	7.8	0.0	7.7	15.1	14.5
Xestospongia sp. 1	10.2	9.3	0.0	8.3	13.9	11.6
P. heteroraphis	13.7	8.0	0.0	8.2	5.2	2.3
Axinyssa sp.	18.6	13.5	12.5	15.5	10.1	3.8
T. cervicornis	9.3	7.8	3.2	3.5	2.9	2.6
H. fascigera	0.0	6.4	0.0	3.6	3.4	2.5
Callyspongia sp.	10.3	8.5	7.8	7.6	15.1	12.3
H. erectus	10.7	8.0	0.0	0.0	8.3	7.9
Xestospongia sp. 2	16.7	8.6	0.0	10.3	11.2	12.4
Cinachyrella sp.	14.1	3.2	0.0	0.0	8.3	7.5
Methanol	0.0	0.0	0.0	0.0	0.0	0.0
Chloramphenicol (30 μg)	25.0	25.7	32.0	26.0	–	–
Amphotericin B (100 μg)	–	–	–	–	21.2	20.3

All twelve sponges extracts displayed inhibitory activities against the Gram-positive bacterium *B. subtilis*. Three extracts of sponges *S. massa*, *Xestospongia* sp.1 and *Axinyssa* sp. showed the highest activities with inhibition zones of 14.3, 9.3 and 13.5 mm, respectively. Eight extracts that showed moderate activity were obtained from *D. fragilis, T. swinhoei, P. heteroraphis, T. cervicornis, H. fascigera, Callyspongia* sp., *H. erectus and Xestospongia* sp. 2. The extract of *Cinachyrella* sp. showed only weak activity. Eleven sponges extracts were active against the Gram-positive bacterium *S. aureus*. Ten extracts obtained from *S. massa, T. swinhoei, Xestospongia* sp. 1, *P. heteroraphis*, *Axinyssa* sp., *T. cervicornis*, *Callyspongia* sp., *H. erectus*, *Xestospongia* sp. 2 and *Cynachyrella* sp. showed high activities, with inhibition zones of 18.1, 10.7, 10.2, 13.7, 18.6, 9.3, 10.3, 10.7, 16.7 and 14.1 mm, respectively. The extract of the sponge *D. fragilis* exhibited moderate activity.

Out of the twelve sponge extracts analyzed, seven extracts (*S. massa*, *T. swinhoei, Xestospongia* sp. 1, *Axinyssa* sp., *H. erectus*, *Callyspongia* sp. and *Xestospongia* sp. 2) showed relatively high activities against the yeast *C. albicans* (Table 2). Furthermore, *in vitro* growth inhibitions of *C. albicans* and *A. niger* were observed in the extracts of four sponges species, *viz. T. swinhoei*, *Xestospongia* sp. 1, *Callyspongia* sp. and *Xestospongia* sp. 2.

The antibacterial properties of *S. massa* and *Axinyssa* sp. and the antifungal activities of *T. swinhoei*, *Xestospongia* sp. 1, *Callyspongia* sp. and *Xestospongia* sp. 2 have been further characterized by determining the MICs using the broth microdilution method (Table 3).

Table 3
MIC (μg/mL) of sponge extracts against selected microorganisms.

Sponge species	Microorganisms					
	S. aureus	*B. subtilis*	*E. coli*	*V. anguillarum*	*C. albicans*	*A. niger*
S. massa	125	125	500	250	250	–
Axynissa sp.	125	250	500	250	500	–
T. swinhoei	–	–	–	–	125	250
Xestospongia sp. 1	–	–	–	–	250	500
Callyspongia sp.	–	–	–	–	250	500
Xestospongia sp. 2	–	–	–	–	500	500
Chloramphenicol	20	24	18	21	–	–
Amphotericin B	–	–	–	–	12	16

4. Discussion

Marine sponges are the oldest multicellular invertebrate organisms on earth having been in existence for more than 600 million years. Sponges are filter feeders with numerous tiny pores on their surface, which allow water to enter and circulate through a series of canals, where microorganisms and organic particles are filtered out and ingested[8]. This morphology, though simple, has enabled sponges to persist through the course of evolution. Marine sponges produce various secondary metabolites as chemical defensive mechanism to protect themselves from predators as well as infectious microorganisms. Many of these compounds have been found to exhibit interesting biomedical and pharmaceutical traits such as antiviral, antitumor, antimicrobial, anti-inflammatory, immunosuppressive, neurosuppressive, neuroprotective, antifouling and general cytotoxic properties.

In the present era, the search for a new generation of antibiotics is increasingly urgent owing to the increased incidence of multiple resistances among pathogenic microorganisms to drugs currently in clinical use. It is ironic that while antibiotic resistance of pathogenic microorganisms is on the rise, the rate of discovery and development of new antibiotic compounds is declining[9,10]. Only two novel antibacterial drugs have been seen entering the market from the last two decades[11]. It is considered that the islands of Indonesia have an extensive cumulative coastline that supports diverse marine ecosystems. A program has been initiated in our laboratory to screen marine invertebrates, especially sponges, as potential sources of drugs.

The first report of antimicrobial activity in sponge extracts

was conducted by Chairman *et al.*[12], who found antimicrobial properties against both Gram-positive and Gram-negative bacteria in 18 out of 31 sponges tested. In the present work, it is observed that 66.7% of 12 marine sponge extracts examined showed antibacterial activities, while 30.0% of these extracts exhibited antifungal activity. The sponge species that were most promising in their production of antimicrobials were *S. massa* and *Axinyssa* sp. that showed inhibitory effects against four tested bacteria and the yeast *C. albicans*. This finding is the first report of antimicrobial activity in two sponge species. In this work, the methanolic extract of the sponges showed a clear inhibitory specificity towards Gram-positive bacteria. It is known that Gram-positive bacteria tend to be much more sensitive to drug action than Gram-negative bacteria[13]. Many, such as *S. aureus*, are of pathological importance, being pervasive in hospital environments. In essence, the discovery of both Gram-positive and Gram-negative bacteria would contribute to the pharmacological arsenal against resistant bacteria.

Based on the evidences from the antifungal assay, *T. swinhoei*, *Xestospongia* sp. 1 and *Xestospongia* sp. 2 appeared to be the most promising in terms of antifungal activity. These sponges were inhibitory to both fungal pathogens *C. albicans* and *A. niger*. The antifungal activity of the extract from *Xestospongia* might be due to the presence of *Xestospongia*-associated natural products (Xestospongiamide) that have been reported to have inhibitory potential against two fungal pathogens *A. niger* and *C. albicans*[14]. Another sponge, *T. swinhoei*, is known to contain the antifungal compounds such as swinhoeiamide A[15] and theonellamide G[16].

Marine sponges collected from the Anambas Islands in Indonesia possess antimicrobial properties. The most promising species in this respect are the sponges *S. massa* and *Axinyssa* sp. These organisms will be subjected to bioassay-guided fractionations to isolate and identify the active antimicrobial compounds. The encouraging biological activities encountered in this study indicated that the Anambas Islands are hosts to a wide array of marine sponges worthy of further investigations.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgments

This work was supported by a research grant from the Coral Reef Rehabilitation and Management Program – Coral Triangle Initiative (COREMAP CTI-LIPI 2016 No. 10876401/ADB LOAN No. 3094-INO), Indonesian Institute of Sciences awarded to Tutik Murniasih and Masteria Yunovilsa Putra.

References

[1] Mehbub MF, Lei J, Franco C, Zhang W. Marine sponge derived natural products between 2001 and 2010: trends and opportunities for discovery of bioactives. *Mar Drugs* 2014; **12**(8): 4539-77.

[2] Ebada SS, Proksch P. The chemistry of marine sponges. In: Fattorusso E, Gerwick WH, Taglialatela-Scafati O, editors. *Handbook of Marine natural products*. Berlin: Springer Netherlands; 2012, p. 191-293.

[3] Blunt JW, Copp BR, Keyzers RA, Munro MH, Prinsep MR. Marine natural products. *Nat Prod Rep* 2014; **31**(2): 160-258.

[4] Perdicaris S, Vlachogianni T, Valavanidis A. Bioactive natural substances from marine sponges: new developments and prospects for future pharmaceuticals. *Nat Prod Chem Res* 2013; doi: 10.4172/2329-6836.1000114.

[5] Sabdono A, Radjasa OK. Microbial symbionts in marine sponges: marine natural product factory. *J Coast Dev* 2008; **11**(2): 57-61.

[6] Putra MY, Murniasih T. Distribution and diversity of marine natural products from Indonesian marine organisms. *J Coast Life Med* 2016; **4**(2): 104-7.

[7] Qaralleh H, Idid SO, Idid SZ, Saad S, Darnis DS, Taher BM, et al. Antifungal and antibacterial activities of four Malaysian sponge species (Petrosiidae). *J Med Mycol* 2010; **20**(4): 315-20.

[8] Kumar MS, Gupta S, Pal AK. Isolation of bioactive proteins from the marine sponge *Spongosorites halichondriodes* (Dendy, 1905). *World Appl Sci J* 2012; **17**(6): 729-34.

[9] Spellberg B. The future of antibiotics. *Crit Care* 2014; **18**(3): 228.

[10] Chellat MF, Raguž L, Riedl R. Targeting antibiotic resistance. *Angew Chem Int Ed Engl* 2016; **55**(23): 6600-26.

[11] Leonard SN, Murphy K, Zaeem M, DiVall MV. An introductory review module for an anti-infectives therapeutics course. *Am J Pharm Educ* 2012; **76**(7): 135.

[12] Chairman K, Ranjit Singh AJA, Ramesh M. Screening twelve species of sponges for biomedical activity in Gulf of Mannar Tuticorin Coast. *Int J of Mar Sci* 2012; **2**(6): 43-50.

[13] Cos P, Vlietinck AJ, Berghe DV, Maes L. Anti-infective potential of natural products: how to develop a stronger *in vitro* 'proof-of-concept'. *J Ethnopharmacol* 2006; **106**(3): 290-302.

[14] Ayyad SE, Katoua DF, Alarif WM, Sobahi TR, Aly MM, Shaala LA, et al. Two new polyacetylene derivatives from the Red Sea sponge *Xestospongia* sp. *Z Naturforsch C* 2015; **70**(11-12): 297-303.

[15] Edrada RA, Ebel R, Supriyono A, Wray V, Schupp P, Steube K, et al. Swinhoeiamide A, a new highly active calyculin derivative from the marine sponge *Theonella swinhoei*. *J Nat Prod* 2002; **65**(8): 1168-72.

[16] Youssef DT, Shaala LA, Mohamed GA, Badr JM, Bamanie FH, Ibrahim SR. Theonellamide G, a potent antifungal and cytotoxic bicyclic glycopeptide from the Red Sea marine sponge *Theonella swinhoei*. *Mar Drugs* 2014; **12**(4): 1911-23.