



Biological activities of extracts from Andaman Sea sponges, Thailand

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Abstract

Thirty six organic extracts were prepared from eighteen marine sponges collected from the Andaman Sea, Thailand. The extracts were examined for anti-malaria, anti-*Microbacterium tuberculosis*, anti-herpes simplex virus, antimicrobial, anti-acetylcholinesterase enzyme and cytotoxic activities. Four extracts showed anti-*M. tuberculosis*, one anti-malarial, twenty four antimicrobial and one extract exhibited cytotoxic activity. However, anti-acetylcholinesterase enzyme and anti-herpes simplex virus type 1 (HSV-1) activities were not recorded. Dichloromethane extracts prepared from *Axinyssa* sp., *Halichondria* sp. and *Chondrosia reticulata* exhibited potential anti-*M. tuberculosis* at MIC 50, 100 and 200 mg/mL, respectively. The Hexane part of *Phakellia ventilabrum* extract showed anti-malarial activity (MIC = 2.8 mg/mL) while the dichloromethane extract showed anti-*M. tuberculosis* and cytotoxic activity with MIC 200 and IC₅₀ 7.1 mg/mL, respectively. Antimicrobial activity was found in both the hexane and dichloromethane parts of extracts.

Keywords: Andaman Sea, anti-malaria, antimicrobial, biological activity, marine sponges.

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INTRODUCTION

Tuberculosis, influenza, AIDS, malaria and cancers are leading dreadful diseases. Resistance to first-line drugs has been observed for those diseases; hence the need for the discovery of new lead molecules (Bredel 2001, Lipsitch et al. 2007, Wright and Zignol 2008). The discovery process for lead molecules starts with the collection of terrestrial or marine organisms, and examines the biological activities of their extracts. This is the most important step providing information for the isolation and purification of those molecules (Faulkner 2000). Among marine organisms, the largest number of secondary metabolites isolated since 1965 have come from sponges (Belarbi et al. 2003), and they have been the primary source of biologically active molecules. The main biological activities of those sponge

metabolites have been cytotoxic and antimicrobial while other activities have been limited, suggesting the need for an evaluation of anti-cancer and anti-infective agents (Mayer and Gustafson 2003, Newman and Cragg 2004). Although many bioactive secondary metabolites have been discovered in sponges, only a few have been commercialized (Belarbi et al. 2003). The most intensively investigated sponges have been those collected from the China Sea, Japan and the West Pacific, followed by those from the Indian Ocean and other regions (Blunt et al. 2009). The West coast of Thailand covers 126,000 km² of the Andaman Sea, which is directly connected to the Indian Ocean. Even though it has a unique

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and diverse sponge fauna, screening of their extracts for biological activity has been rare. Therefore, we set out to determine the biological activities (both anti-infection and cytotoxic) of 36 organic extracts prepared from sponges collected from the Andaman Sea, Thailand.

MATERIAL AND METHODS

Marine sponge collection: Marine sponges were collected by scuba divers at 10-20 m deep along the Andaman Sea coast (Trang and Krabi Provinces). Samples were kept in sealed plastic packs in ice boxes before freezing at -20°C until analysis. Voucher specimens were preserved in 75% methanol and deposited at the Department of Marine Science, Rajamangala University of Technology Srivijaya (RMUSTV), Trang Campus.

Sponge identification: Sponges were identified by one of us (UD) using the manual key of Hooper and Soest (2002).

Preparation of the extracts: Samples (1 kg wet weight) were repeatedly macerated in methanol (6 L each). The extract was filtered out and the organic solvent was removed by vacuum. The aqueous methanol was further partitioned with hexane and dichloromethane (DCM), respectively. The completion of extraction was routinely checked by thin layer chromatography (TLC) before partitioning with the higher polar organic solvent.

Biological activities determination

Anti-malaria: Plasmodium falciparum (K1, multi drug resistant strain) was cultured in vitro in RPMI 1640 medium in a humidified 37°C incubator with 3% CO_2 as proposed by Trager and Jensen (1976). Quantitative assessment of anti-malarial activity in vitro was determined by microculture radioisotope techniques based upon the method described by Desjardins et al (1979). The anti-malarial agents, dihydroartemisinin and 0.1% DMSO were used as the positive and negative control, respectively. The IC_{50} values of tested extracts and controls were determined by logarithmic dose-response curves and expressed as a percentage of the parasite-

specific inhibitor ^3H -hypoxanthine incorporated. Samples showing IC_{50} values <10 mg/mL were considered as strongly active.

Anti-tuberculosis: Anti-*M. tuberculosis* strain H37Ra activity was assessed in 384-well plates by the GFP method (Changsen et al. 2003). The maximum concentration of the tested samples was $50\ \mu\text{g/mL}$. The assay was performed in duplicate with each well containing $5\ \mu\text{L}$ of test sample which was serially diluted in 5% dimethyl sulfoxide, followed by $45\ \mu\text{L}$ of cell suspension (approximately, 2×10^4 to 1×10^5 cfu/mL/well). The plates were cultured in a humidified incubator at 37°C for 7 d before measuring fluorescence signals using a SpectraMax M5 microplate reader (Molecular Devices, USA). The minimum inhibitory concentration (MIC) was defined as the lowest concentration affecting a reduction in fluorescence of 90% relative to controls. Rifampicin, streptomycin, isoniazid and ofloxacin were used as positive controls, while 0.5% DMSO was used as a negative control.

Anti-herpes simplex virus: Anti-herpes simplex virus type 1 (HSV-1) activity was tested against HSV-1 strain ATCC VR 260 (preserve at NSTDA Bangkok, Thailand) using a colorimetric microtiter plate assay that determined growth of the host cell by measuring cellular protein content as described by Skehan et al. (1990). Host cells (Vero cell line ATCC CCL-81) were infected with virus and treated with extract (final concentration was $50\ \mu\text{g/mL}$) which was compared with control cells (infected with virus only). Acyclovir and DMSO were used as the positive and negative control, respectively. Extracts that inhibit virus growth more than 50% were further examination to determine IC_{50} .

Cytotoxicity: Cytotoxicity against Vero cell (African green monkey kidney cell, ATCC CCL-81) was determined by the green fluorescent protein (GFP) detection method (Hunt et al. 1999). The assay was carried out by adding $45\ \mu\text{L}$ of cell suspension at 3.3×10^4 cell/mL to 384-well plates

containing 5 μ L of test compounds previously diluted in 0.5% DMSO (maximum concentration was 50 μ g/mL). After 4 d of humidified incubation at 37°C with 5% CO₂, fluorescence signals were measured using a Spectra Max M5 microplate reader (Molecular Devices, USA) in the bottom reading mode with excitation and emission wave lengths of 485 and 535 nm. The IC₅₀ values were derived from dose-response curves by using 6

concentrations of 2-fold serially diluted samples with the SOFTMax Pro software (Molecular device). Ellipticine and 0.5% DMSO were used as the positive and negative control, respectively.

Anti-acetylcholinesterase: By using TLC with combining bioassay for acetylcholinesterase (AChE) inhibitor described by Rhee et al. (2001), the extracts were dissolved in DCM and applied on silica gel TLC plates

Table 1. Biological activities of extracts from marine sponges.

SI No.	Name of sponge**	Part of extract	Types of biological activity*					
			Mt	ct	am	Sa ^a	Bs ^a	MI ^a
1.	<i>Ptilocaulis</i> sp. ^b (LAN-06-07)	hexane	-	-	-	11	-	11
		DCM	-	-	-	-	-	11
2.	<i>Axinyssa</i> sp. ^b (LAN-06-21)	hexane	-	-	-	-	-	18
		DCM	-	-	-	-	-	-
3.	<i>Axinyssa</i> sp. ^b (LAN-06-28)	hexane	-	-	-	10	-	-
		DCM	-	-	-	-	-	10
4.	<i>Axinyssa</i> sp. ^b (POR-06-03)	hexane	-	-	-	-	-	-
		DCM	50	-	-	-	-	-
5.	<i>Axinella</i> sp. ^b (LAN-06-26)	hexane	-	-	-	10	-	-
		DCM	-	-	-	-	-	-
6.	<i>Chondrosia reticulata</i> Carte, 1886 (LAN-06-11)	hexane	-	-	-	-	-	-
		DCM	200	-	-	-	-	22
7.	<i>Chondrosia</i> sp. ^b (LAN-06-23)	hexane	-	-	-	-	-	10
		DCM	-	-	-	-	-	-
8.	<i>Gelliodes petrosioides</i> Dendy, 1905 (LAN-06-03)	Hexane	-	-	-	-	-	12
		DCM	-	-	-	-	-	-
9.	<i>Halichondria</i> sp. ^b (POR-06-01)	hexane	-	-	-	-	-	-
		DCM	100	-	-	-	-	10
10.	<i>Ircinia mutans</i> Wilson, 1925 (LAN-06-29)	hexane	-	-	-	-	-	-
		DCM	-	-	-	-	-	8
11.	<i>Oceanapia</i> sp. ^b (LAN-06-20)	hexane	-	-	-	-	-	-
		DCM	-	-	-	10	-	-
12.	<i>Phakellia ventilabrum</i> Linnaeus, 1767 (LAN-06-17)	hexane	-	-	2.8	10	10	11
		DCM	200	7.1	-	10	-	-
13.	<i>Psammoclema ramosum</i> Marshall, 1880 (POR-06-04)	hexane	-	-	-	-	-	-
		DCM	-	-	-	-	-	10
14.	<i>Ptilocaulis</i> sp. ^b (LAN-06-07)	hexane	-	-	-	10	-	-
		DCM	-	-	-	-	-	-
15.	<i>Theonella</i> sp. ^b (LAN-06-12)	hexane	-	-	-	-	-	-
		DCM	-	-	-	12	-	-
16.	<i>Sigmosceptrella</i> sp. ^b (POR-06-02)	hexane	-	-	-	12	-	-
		DCM	-	-	-	-	-	10
17.	<i>Xestospongia</i> sp. ^b (LAN-06-24)	hexane	-	-	-	-	-	-
		DCM	-	-	-	-	-	10
18.	<i>Xestospongia</i> sp. ^b (POR-06-05)	hexane	-	-	-	-	-	-
		DCM	-	-	-	12	-	-
		rifampicin	0.01	-	-	-	-	-
		streptomycin	0.3	-	-	-	-	-
		isoniazid	0.1	-	-	-	-	-
		ofloxacin	0.8	-	-	-	-	-
ellipticine	-	1.8	-	-	-	-		
dihydroartemisinin	-	-	4.60 nM	-	-	-		
norfloxacin	-	-	-	-	24	22	21	

Mt = anti-*M. tuberculosis* was recorded as MIC values (μ g/mL). ct = cytotoxicity against Vero cell and anti-malaria (am) were recorded as IC₅₀ values (μ g/mL). ^aAntimicrobial activity was recorded as zone of inhibition (mm). Sa =

Staphylococcus aureus, Bs = *Bacillus subtilis*, MI = *Micrococcus luteus*. ^bHitherto undescribed species.

*Anti-HSV-1, anti-acetylcholinesterase enzyme and antimicrobial against *E. coli* were no observed.

**The sample codes are in parenthesis.

which were further developed in a selected mobile phase (hexane: DCM, 2:8, v/v) before spraying with 30 mmol of acetylcholine iodide followed by 20 mmol of 5, 5'-dithiobis (2-nitrobenzoic acid). After 45 min the TLC plates were sprayed with 10.2 $\mu\text{g}/\text{mL}$ TcAChE. The TLC plates had a yellow background with white spots of AChE inhibitory compounds.

Antimicrobial: Antimicrobial activity was determined by the modified agar diffusion method (Traub et al. 1998). The test organisms (*Bacillus subtilis*, ATCC 6633; *Staphylococcus aureus*, ATCC 25923; *Micrococcus luteus*, ATCC 9341 and *Escherichia coli*, ATCC 35218) were prepared to a standard density of McFarland number 0.5 before spreading on the agar. The extracts were dissolved in DCM and transferred to test discs at the final concentration of 20 $\mu\text{g}/\text{disc}$, let dry before laying on the agar, and incubated at 37°C for 12 h. Norfloxacin (10 $\mu\text{g}/\text{disc}$) and 5% DMSO were used as the positive and negative control, respectively. The zone of inhibition was measured in millimeters.

RESULTS

The biological activities of the extracts from marine sponges are provided in Table 1. Four extracts showed activity that was anti-*M. tuberculosis*, one cytotoxic and anti-malarial, and twenty four that were antimicrobial. Anti-*M. tuberculosis* activity was found in DCM extracts of *Chondrosia reticulata*, *Phakellia ventilabrum*, *Halichondria* sp. and *Axinyssa* sp. (POR-06-03). DCM and a hexane extract of *Phakellia ventilabrum* also showed cytotoxic and anti-malarial activity, respectively, while antimicrobial activity was found in both DCM and hexane part of extracts. None of the extracts showed anti-HSV-1, antimicrobial against *E. coli* or anti-acetylcholinesterase enzyme activities.

DISCUSSION

Research has indicated that the secondary metabolites of sponges play an important role in their defense against infectious microorganisms (Proksch 1994). That view was supported by the finding that all sponge extracts had the capability to inhibit at least one strain of pathogenic microorganism (Table 1). Sponges collected from the Caribbean and Tunisian Sea regions (Galeano and Martínez 2007, Touati et al. 2007) that were extracted by intermediate polar solvents, such as chloroform and ethyl acetate, inhibited both gram-positive and gram-negative bacteria. In contrast, sponge extracts from our study were moderately active against only gram-positive bacteria. These results led us to conclude that the extracts of Andaman Sea sponges do not contain broad-spectrum antimicrobial substances, and furthermore, that the cell walls of gram-positive bacteria are more highly sensitive than gram-negative bacteria to attack by antimicrobial agents, because of the teichoic acid assembly in their structure (Duguid 1965). However, some extracts from specific taxa, including the DCM part of *Chondrosia reticulata* and the hexane part of *Axinyssa* sp. (LAN-06-21), showed highly potent activity against *M. luteus*, with zones of inhibition about 22 and 18 mm, respectively. This could have been because of the presence of long-chain antibacterial fatty acids, particularly 8, 10, Me2-16:0 (Nechev 2002, Rodkina 2005) in the genus *Chondrosia* spp., and strong antibacterial germacranes sesquiterpene in the genus *Axinyssa* sp. (Satitpatipan and Suwanborirux 2004). The DCM extract of *Phakellia ventilabrum* showed both strong cytotoxic activity against Vero cell and moderately anti-infection activity, which reflected the presence of typical cytotoxic cyclic peptides in the extract (Pettit et al. 1994, Li et al. 2003, Pettit and Tan 2005). Even though the genus *Phakellia* sp. produces several classes of biologically active

secondary metabolites, such as alkaloids, sterols, acetylenic acid, peptide and polyether acid (Xu et al. 2003), extracts from it did not show anti-infection activity.

Some extracts of sponges are promising for further isolation of anti-infection and cytotoxic secondary metabolites, particularly *Axinyssa* spp., *Halichondria* sp., *Phakellia ventilabrum* and *Chondrosia reticulata*.

CONCLUSION

Biological activity was examined in 36 sponge extracts collected from the Andaman Sea. The results indicated selective biological activity against *M. tuberculosis* (11.1%), cytotoxic activity against Vero cells (2.8%), anti-malarial (2.8%) and antimicrobial activity (66.7%), thus showing that Andaman Sea sponges provide an important source of biologically active secondary metabolites.

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Tayland Andaman Denizi Süngerlerinden Elde Edilen Özütlerin Biyolojik Aktivitesi

Özet

Tayland'in Andaman Denizi'nden toplanan on sekiz deniz süngerinden otuz altı organik özüt hazırlandı. Özütlerin; anti-sitma, anti-*Microbacterium tuberculosis*, anti-uçuk (*Herpes simplex*) virüsü, antimikrobiyal, anti-asetilkolinesteraz ve sitotoksik aktiviteleri araştırıldı. Özütleri dördü anti-*M. tuberculosis*, biri anti-sitma, yirmi dördü antimikrobiyal ve bir tanesi de sitotoksik etki gösterdi. Ancak, herhangi bir anti-asetilkolinesteraz ve anti-uçuk (*Herpes simplex* tip 1 (HSV-1)) virüsü aktivitesi görülmedi. *Axinyssa* sp., *Halichondria* sp. ve *Chondrosia reticulata*'dan hazırlanan diklorometan özütleri, sırasıyla MIC 50, 100 ve 200 mg/mL'de potansiyel anti-*M. tuberculosis* etki gösterdi. *Phakellia ventilabrum*'un hekzan kısmı (MIC = 2.8 mg/mL) anti-sitma özellik sergilerken, diklorometan özütleri sırasıyla MIC 200 ve IC₅₀ 7.1 mg/mL'de anti-*M. tuberculosis* ve sitotoksikite gösterdi. Özütlerin hem hekzan hem de diklorometan kısımlarında antimikrobiyal aktivite tespit edilmiştir.

Anahtar Kelimeler: Andaman Denizi, anti-sitma, antimikrobiyal, biyolojik aktivite, deniz süngerleri.