

Identification of Antibacterial Activity with Bioactive Compounds from Selected Marine Sponges

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ABSTRACT

Background: Marine sponges (phylum Porifera) are sessile filter-feeders from the ocean that are becoming the wealthiest sources of pharmacologically active compounds. **Objectives:** Our objectives are to identify bioactive compounds from marine sponges (*Xestospongia exigua*, *Xestospongia muta*, and *Iotrochota baculifera*) and to determine their antibacterial activity. **Materials and Methods:** Methanolic crude extracts were subjected to two-steps fractionation: first, solvent partitioning was conducted using diethyl ether and butanol, followed by column chromatography. The resulting fractions were tested for antibacterial activity against four bacterial strains (*Staphylococcus aureus* ATCC 25923, *Micrococcus luteus* ATCC 4698, *Escherichia coli* ATCC 11775, and *Salmonella typhimurium* ATCC 14128). The fractions were subsequently profiled using High-Performance Thin Layer Chromatography (HPTLC), and the component of active sub-fractions (SF) was identified using Gas Chromatography-Mass Spectrometry (GC-MS). **Results:** Although no antibacterial activity was recorded of the methanolic extracts in all marine sponges samples, the response towards diethyl ether extracts of *X. exigua* was strong. Out of 17 sub-fractions of diethyl ether profiled, three sub-fractions, i.e. 5, 13, and 14 were active. GC-MS identified five compounds in SF 5, four compounds in SF 13, and three compounds in SF 14. Furthermore, SF 13 and SF 14 could inhibit the growth of all bacteria tested, indicating a broad-spectrum activity. On the contrary, SF 5 showed selective inhibition only to *E. coli* and *S. typhimurium*, indicating narrow-spectrum activity. **Conclusion:** Bioactive SF 13 of *X. exigua* has a high potential as an antibacterial agent but in vitro assessment such as cytotoxicity against mammalian cell lines is needed to determine the toxicity and drug response.

Key words: Diethyl ether, HPTLC, *Iotrochota baculifera*, Minimum Inhibitory Concentration, *Xestospongia exigua*, *X. muta*.

INTRODUCTION

As the world evolves, new contagious diseases emerged. To date, the bacterial infection has become an issue of concern to health practitioners requiring an effective intervention strategy to curb the widespread use of antibiotics.¹ Antibiotic resistance is the biggest challenge in today's global health since the misuse or overuse of antibiotics causes the infections to be much harder to treat. The World Health Organization (WHO) has declared ten species of bacterial strains that can develop into multi-resistant antibiotic strains. They are *Streptococcus pneumoniae*, *Klebsiella pneumoniae*, *Mycobacterium tuberculosis*, *Neisseria gonorrhoeae*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Escherichia coli*, *Enterococcus faecium*, and *Enterococcus faecalis*.²

Marine invertebrates are becoming an essential source of novel bioactive compounds for the treatment of infectious diseases. The reasons are due to the long history of evolution, and its molecular diversity is more advanced relative to terrestrial equivalents.³ Marine sponges are a type of sessile aquatic invertebrate animal, which belongs to the phylum Porifera.⁴ They have no symmetrical tissues

or organs and can be found in various shapes, sizes, and colours. Numerous tiny spores and canals can be found on their bodies that functionally involved in the filter-feeding system.⁵ Most of the sponges that originated from the South China Sea belong to the class Demospongiae. Ten orders were identified in this class; among them are axinellida, hadromerida, holichondria, haplosclerida, and homosclerophoria, with a total of 29 families and more than 121 species.⁶

To date, around 5300 compounds have been successfully isolated from 500 species of sponges.⁷ Each compound has been reported as having antibacterial, anticancer, anti-fungal, anti-tuberculosis, anti-inflammatory, anti-fouling, and anti-malarial properties.^{8,9} Due to its species-richness, sponges are regarded as a prolific source of novel steroids, terpenoids, peptides, macrolides, and alkaloids.¹⁰ Most of these biologically active compounds have unique, unprecedented structures with cyclic or linear peptides containing the unusual amino acid, as compared to compounds from other sources such as terrestrial and microbial systems.¹¹ In this study, the bioactive compounds from three marine sponges (*X. exigua*, *X. muta*, and *I. baculifera*) were identified and their antibacterial activity was determined.

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MATERIALS AND METHODS

On-site field sampling

Samples of marine sponges (*X. exigua*, *X. muta*, and *I. baculifera*) were collected from Bidong and Karah Islands in Terengganu by scuba diving at a depth of 8 to 15 m. The details of the collection sites are shown in Table 1. The samples were identified and authenticated taxonomically by experts. After collection, the samples were stored on ice and transported to the laboratory at the Institute of Marine Biotechnology, Universiti Malaysia Terengganu (UMT). Then, the samples were subjected to lyophilisation using a freeze-dryer machine (Labconco, USA) and later ground to obtain the powder.

Preparation of marine sponges extracts

The lyophilised sample was extracted using the cold maceration technique.¹² Briefly, 10 g of each sample of *X. exigua*, *X. muta*, and *I. baculifera* were soaked into absolute methanol at 10% (w/v). The soaking step was repeated three to four times at room temperature overnight to maximise the extraction yield. The extracts were filtered out the following day using a Whatman filter paper (No. 2). Next, the supernatant was concentrated using a rotary evaporator (Buchi, USA) to obtain the methanol crude extract.

Separation of crude extracts using solvent partitioning

Crude extracts of the marine sponges were separated using liquid-liquid extraction by two-phases of organic solvents separation comprising of diethyl ether and butanol, which were chosen based on their polarities, with some modifications.¹³ First, the sample was immersed in diethyl ether. After that, the samples were transferred into the separating funnel. Next, distilled water was added into the diethyl ether extract at a ratio of 1:2. The mixture was shaken vigorously to settle down the compounds. The partitioning process was repeated at least three times until the diethyl ether phase turned colourless. After the separation process was completed, the organic layer consisting of diethyl ether was removed so that the aqueous layer remained for the second separation process using butanol as an organic solvent. After the addition of butanol into the aqueous layer, distilled water was subsequently added to obtain a mixture of polar and non-polar phases. The mixture was shaken vigorously, and the polar and non-polar fractions were concentrated using a rotary evaporator (IKA, Taiwan). The extracts were stored at room temperature until further use for fractionation.

Metabolites profiling using high-performance thin layer chromatography (HPTLC)

The metabolites constituents of the extracts were profiled using HPTLC, following the method described by Minase and Dole¹⁴ with some modifications. The HPTLC (Camag, Switzerland) was performed on a pre-coated HPTLC plate of silica gel 60 F₂₅₄ (Merck, Germany) at 20 cm × 10 cm, with a thickness of 200 µm. The sub-fractions were reconstituted in methanol at a concentration of 10 mg/mL. Then, the samples were applied on the plate under a mixture of a solvent system consisting of hexane: ethyl acetate at a ratio of 7:3. After running the

extracts through the plate using the complete solvent system, the plate was visualised under two different UV wavelengths at 254 nm and 366 nm following the method described by Ebada *et al.*¹⁵ Both UV wavelengths were selected because they could detect the presence of aromatic rings, conjugated double bonds, and unsaturated compounds. Next, the derivatisation of compounds such as phenols, terpenes, sugars, steroids, amines aldehydes, ketones, or allylic alcohols in the samples was further detected by using p-anisaldehyde-sulphuric acid reagent as a visualising agent.

Identification of compounds by gas chromatography-mass spectrometry (GC-MS)

The compounds present in the samples were identified using GC-MS (Shimadzu, Japan). The apparatus for GC-MS consisted of a one-fused silica capillary column made up of 100% dimethyl polysiloxane at the dimension size of 30 mm (length) × 0.25 mm (diameter) × 0.25 mm (film thickness). Helium gas at 99.9% saturation was injected at a flow-rate of 0.96 mL/min to permit electron ionisation energy to occur at 70 eV. The temperatures of the injector and ion sources were maintained at 300 °C. The oven temperature was initially at 40 °C for 2 min, then increased at a rate of 9 °C/min until 300 °C and held at 300 °C for 3 min. Mass spectra were recorded within a scan-interval at 0.5 s and scan range at 50–600 m/z. The total GC running time was 56 min. The percentage of each component was calculated by comparing its average peak area to the total area following the method described by Anupriya *et al.*¹⁶ The similarity index of the major compounds detected were compared with the National of Standards and Technology (NIST), Chemistry Web-book, and Golm Metabolome Database (GMD).

Determination of antibacterial activity using disc diffusion test (DDT)

The protocol for DDT was performed according to the method described by Laila *et al.*¹⁷ with some modifications. Two test strains of Gram-positive bacteria (*S. aureus* [ATCC 25923] and *Micrococcus luteus* [ATCC 4698]) and two test strains of Gram-negative bacteria (*E. coli* [ATCC 11775] and *Salmonella typhimurium* [ATCC 14128]) were used. All the test strains were obtained from the Microbiology Laboratory, Universiti Malaysia Terengganu. The inoculum of test strains was cultured into the nutrient broth. The solutions were vortexed to form a smooth suspension and subjected to incubation overnight. For the DDT assay, approximately 20 mL of sterile Mueller Hinton Agar (MHA) was poured into the Petri dishes and allowed to sit at room temperature. Then, the turbidity of the overnight culture of test strains was adjusted to a 0.5 McFarland standard. After adjustment, the test strains were swabbed uniformly on the solidified agar using a cotton swab and allowed to dry for about 10 min. Sterilised discs were then impregnated with samples and carefully placed onto the agar plate by using sterilised forceps. The negative control was prepared using methanol, while ampicillin was used as a positive control. The reference standard for the measurement of inhibition zones was performed according to Chandra *et al.*¹⁸

Determination of half inhibitory concentration (IC₅₀) using broth microdilution method (BMM)

The protocol of BMM was performed on a 96-well microtiter plate based on Kannan *et al.*¹⁹ with some modifications. Each sample of diethyl ether sub-fractions (SF 5, SF 13, and SF 14) was diluted at the concentrations of 500, 250, 125, and 62.5 µg/mL in a microcentrifuge tube. The turbidity of the bacterial suspension was adjusted to 0.5 McFarland standard to produce 1 × 10⁵ CFU/mL. To start the BMM assay, 50 µL Mueller Hinton broth (MHB) containing each bacterial test strains (*S. aureus*, *M. luteus*, *E. coli*, and *S. typhimurium*) were added into the microtiter plate as control. For the blank, 100 µL of MHB was added into the well.

Table 1: Description of location of sampling site for selected marine sponges.

Sample	Location of Sampling	Global Positioning System
<i>X. exigua</i>	Bidong Island	N 05°37.6306' E 103°31.7874'
<i>X. muta</i>	Bidong Island	N 04°12.8000' E 100°32.5900'
<i>I. baculifera</i>	Karah Island	N 05°35.5000' E 103°33.4000'

Meanwhile, for the test samples, 100 μ L bacterial test strains from each sample (*S. aureus*, *M. luteus*, *E. coli*, and *S. typhimurium*) were added into the wells containing 100 μ L of each diethyl ether sub-fractions (SF 5, SF 13, and SF 14) at the respective concentrations (500, 250, 125, and 62.5 μ g/mL). Next, the microtiter plates were incubated at 37 °C for 24 hr. The bacterial colonies were counted the following day using the GloMax microplate reader (Promega, UK) at the absorbance of 595 nm. The absorbance values were converted into growth inhibition percentage using the formula stated below. Half-maximal inhibitory concentration (IC_{50}) was obtained graphically from the dose-response curves.

$$\text{Inhibition (\%)} = (\text{NC} - \text{T}) / \text{NC} \times 100$$

NC = Negative Control

T = Sample Test

RESULTS

Metabolite profiles

Based on the HPTLC chromatogram, Figure 1(a) demonstrates the visible compounds of *X. exigua* under the short UV wavelength showing ten spots of dark colour at eight different sub-fractions (number 2, 4, 5, 10, 12, 13, 14, and 15). Meanwhile, Figure 1(b), which illustrates the results of the long UV wavelength, shows seven blue spots observed at seven different sub-fractions (number 2, 3, 4, 5, 6, 7, and 9). The mixture of blue and red spots was visible at eight different sub-fractions, including SF 10, SF 11, SF 12, SF 13, SF 14, SF 15, SF 16, and SF 17. Both short- and long-UV wavelengths can detect the compounds with aromatic rings or conjugated double bonds as well as some unsaturated compounds such as polyphenols. After being derivatised by p-anisaldehyde, as shown in Figure 1(c), the spots are relatively more abundant at all sub-fractions as compared to the number of spots in the short- and long-UV wavelengths, indicating the presence of compounds corresponding to phenols, terpenes, sugars, steroids, allylic alcohols, amines, aldehydes, or ketones.

Composition of identified compounds

Identification of compounds using GC-MS revealed that only three sub-fractions were active, consisting of SF 5, SF 13, and SF 14 (Table 2). Five

major compounds were identified at SF 5 with the maximum area of the peak recorded by octadecanoic acid butyl ester (5.38%), 1-eicosanol (2.90%), bis(2-ethylhexyl) phthalate (2.51%), eicosanoic acid-2-hydroxyethyl ester (2.32%), and hexadecanoic acid butyl ester (1.96%; as shown in Table 2). The highest percentage of compounds present in SF 5 are hexadecanoic acid butyl ester (51.0%) as compared to octadecanoic acid butyl ester, which is present at the lowest percentage (18.6%). All three sub-fractions (SF 5, SF 13, and SF 14) showed the presence of bis(2-ethylhexyl) phthalate with the highest percentage in SF 5 at 39.8%, followed by SF 13 (33.1%), and SF 14 (17.8%). Octadecanoic acid butyl ester is present only in two sub-fractions (SF 5 and SF 13) at an equal percentage (18.6%) but is absent in SF 14. As for SF 13, four compounds are present including bis(2-ethylhexyl) phthalate contributing to the major compounds (33.1%), followed by octadecanoic acid butyl ester (18.6%), n-hexadecanoic acid (8.5%), and 2-tert-butyl-4,6-bis(3,5-di-tert-butyl-4-hydroxybenzyl)phenol (3.4%; Table 2). Meanwhile, three compounds were identified in SF 14, with the major component being hexanedioic acid bis(2-ethylhexyl) ester, followed by bis(2-ethylhexyl) phthalate, and 9-19-cycloplanostan-3-ol-acetate (3 β).

Antibacterial activity of marine sponges extract against ATCC bacteria test strains

Antibacterial susceptibility test using DDT showed that *X. exigua* moderately inhibited the growth of *S. aureus* and *E. coli*. However, no activity was observed towards *M. luteus* and *S. typhimurium* (Table 3). Two other marine sponges (*X. muta* and *I. baculifera*) gave a moderate response only to *M. luteus* but no activity to other test strains (Table 3). All bacterial test strains are strongly inhibited when incubated with the diethyl ether fraction of *X. exigua* (DEF; Table 4). In contrast, the butanol fraction of *X. exigua* showed a moderate inhibition only to *M. luteus* but not to any other strains. Out of the three active sub-fractions, two sub-fractions (SF 13 and SF 14) of *X. exigua* gave a positive response to all bacterial strains (*S. aureus*, *M. luteus*, *E. coli*, and *S. typhimurium*; Table 5). Both SF 13 and SF 14 have strongly inhibited the growth of *M. luteus* and *S. typhimurium* but gave a weak activity against *S. aureus* and *E. coli* (Table 5). As for SF 5, a strong activity is shown against *S. typhimurium* (Table 5), but the activity of this sub-fraction has weakly inhibited the growth of *E. coli*. There is no activity detected for SF 5 against *S. aureus* and *M. luteus*.

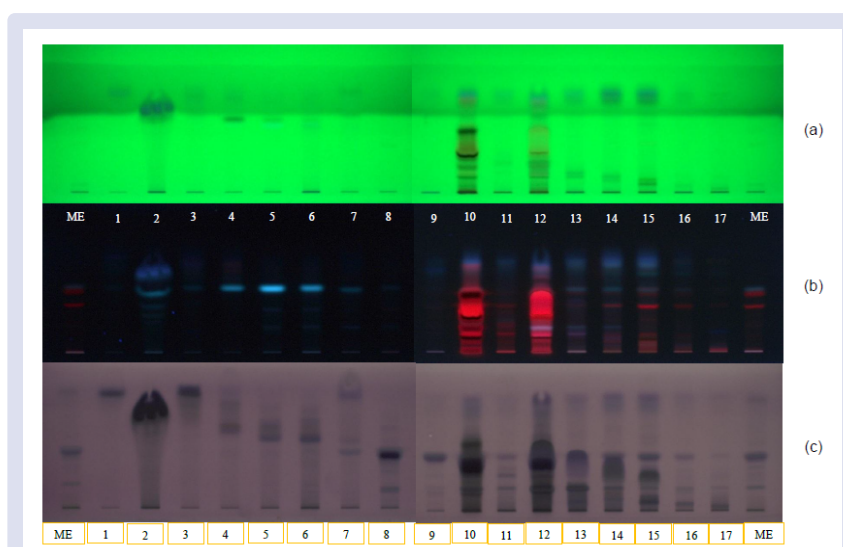


Figure 1: HPTLC chromatogram of 17 sub-fractions from diethyl ether fractions of *X. exigua* developed under (a) short UV wavelength (254 nm), (b) long UV wavelength (366 nm), and (c) derivatised by using p-anisaldehyde-sulphuric acid reagent. Abbreviation ME indicates methanolic extract.

Table 2: Identified components from diethyl ether sub-fractions 5, 13 and 14 of *X. exigua* using GC-MS analysis.

Sub-fraction	Compounds	Retention Time (min)	Area (%)	Height (%)	Abundance (%)
5	1-eicosanol	36.72	2.90	4.59	34.4
	Hexadecanoic acid butyl ester	38.63	1.96	2.82	51.0
	Octadecanoic acid butyl ester	42.20	5.38	7.87	18.6
	Eicosanoic acid-2-hydroxyethyl ester	43.14	2.32	3.33	43.1
	Bis(2-ethylhexyl)phtalate	44.61	2.51	3.81	39.8
13	n-hexadecanoic acid	34.30	11.73	9.20	8.5
	Octadecanoic acid butyl ester	42.22	5.37	8.06	18.6
	Bis(2-ethylhexyl)phtalate	44.61	3.03	5.59	33.1
	2-tert-butyl-4,6-bis(3,5-di-tert-butyl-4-hydroxybenzyl) phenol	54.19	29.37	20.68	3.4
14	Hexanedioic acid, bis(2-ethylhexyl) ester	42.24	25.21	30.72	4.0
	Bis(2-ethylhexyl) phtalate	44.62	5.64	7.49	17.8
	9-19-cycloplanostan-3-ol-acetate (3 β)	55.67	11.32	4.58	12.9

Table 3: Antibacterial susceptibility test using DDT for *X. exigua*, *X. muta* and *I. baculifera* against four bacteria test strains (*S. aureus*, *M. luteus*, *E. coli* and *S. typhimurium*).

Sample	Antibacterial susceptibility test			
	<i>S. aureus</i>	<i>M. luteus</i>	<i>E. coli</i>	<i>S. typhimurium</i>
<i>X. exigua</i>	M	-	M	-
<i>X. muta</i>	-	W	-	-
<i>I. baculifera</i>	-	W	-	-
Positive control (Ampicillin)	M	S	M	M
Negative control (Methanol)	-	-	-	-

*Degree of inhibition zones was measured in reference to Chandra *et al.*^[21]

{W: weak activity (6-9 mm), M: moderate activity (10-15 mm), S: strong activity (≥ 16 mm), "-": No activity}.

Table 4: Antibacterial susceptibility test using DDT for diethyl ether fraction (DEF) and butanol fraction (BUF) of *X. exigua* against four bacteria test strains (*S. aureus*, *M. luteus*, *E. coli* and *S. typhimurium*).

Sample	Antibacterial susceptibility test			
	<i>S. aureus</i>	<i>M. luteus</i>	<i>E. coli</i>	<i>S. typhimurium</i>
DEF	S	S	S	S
BUF	-	M	-	-
Positive control (Ampicillin)	S	S	S	S
Negative control (Methanol)	-	-	-	-

*Degree of inhibition zones was measured in reference to Chandra *et al.*^[21]

{W: weak activity (6-9 mm), M: moderate activity (10-15 mm), S: strong activity (≥ 16 mm), "-": No activity}.

Table 5: Antibacterial susceptibility test using DDT for diethyl ether sub-fractions 5, 13 and 14 of *X. exigua* against four bacteria test strains (*S. aureus*, *M. luteus*, *E. coli* and *S. typhimurium*).

Sample	Antibacterial susceptibility test			
	<i>S. aureus</i>	<i>M. luteus</i>	<i>E. coli</i>	<i>S. typhimurium</i>
Sub-fraction 5	-	-	W	S
Sub-fraction 13	W	S	W	S
Sub-fraction 14	W	S	M	S
Positive control (Ampicillin)	M	S	M	S
Negative control (Methanol)	-	-	-	-

*Degree of inhibition zones was measured in reference to Chandra *et al.*^[21]

{W: weak activity (6-9 mm), M: moderate activity (10-15 mm), S: strong activity (≥ 16 mm), "-": No activity}.

Half maximal inhibitory concentration (IC_{50})

The minimal concentration of diethyl ether SF 5 of *X. exigua* (62.5 $\mu\text{g/mL}$) managed to inhibit the growth of *M. luteus* at the highest percentage (18%) as compared to other strains of bacteria (*S. aureus*, *E. coli*, and *S. typhimurium*), which are only inhibited at 7%, 13%, and 14%, respectively (Figure 2a). Although a two-fold concentration increase of diethyl ether SF 5 (125 $\mu\text{g/mL}$) inhibited three strains of bacteria (*M. luteus*, *E. coli*, and *S. typhimurium*) at 20%–30% inhibitions, it minimally inhibited *S. aureus* (Figure 2a). Moreover, the detection of a high percentage (70%–75%) of *S. typhimurium* inhibition is in parallel with the increasing concentration of diethyl ether of SF 5. The concentration of this fraction is between 250 $\mu\text{g/mL}$ to 500 $\mu\text{g/mL}$ (Figure 2a), surpassing other bacterial strains growth inhibition (*S.*

aureus: 30% to 45%, *M. luteus*: 45% to 59%, and *E. coli*: 60% to 72%). Both diethyl ether of SF 13 and SF 14 showed the highest antibacterial activity (97% and 92%, respectively) against *M. luteus* at 500 $\mu\text{g/mL}$ (Figure 2b and 2c). However, at the low concentration of 62.5 $\mu\text{g/mL}$, both sub-fractions of SF 13 and SF 14 gave inhibition at 60% and 57%, respectively (Figure 2b and 2c). Similarly, the degree of inhibition increased by the increased concentrations of diethyl ether of SF 13 and SF 14 (62.5 $\mu\text{g/mL}$ to 500 $\mu\text{g/mL}$) in other bacterial strains such as *S. aureus*, *E. coli*, and *S. typhimurium*. Among the three sub-fractions, diethyl ether of SF 13 showed the best potency with the lowest values of IC_{50} to inhibit the growth of *S. aureus*, *M. luteus*, and *S. typhimurium* (561.2 \pm 11.2, 41.7 \pm 2.5 and 106.3 \pm 4.2, respectively; Figure 3). However, for *E. coli*, the diethyl ether of SF 14 produced the best potency,

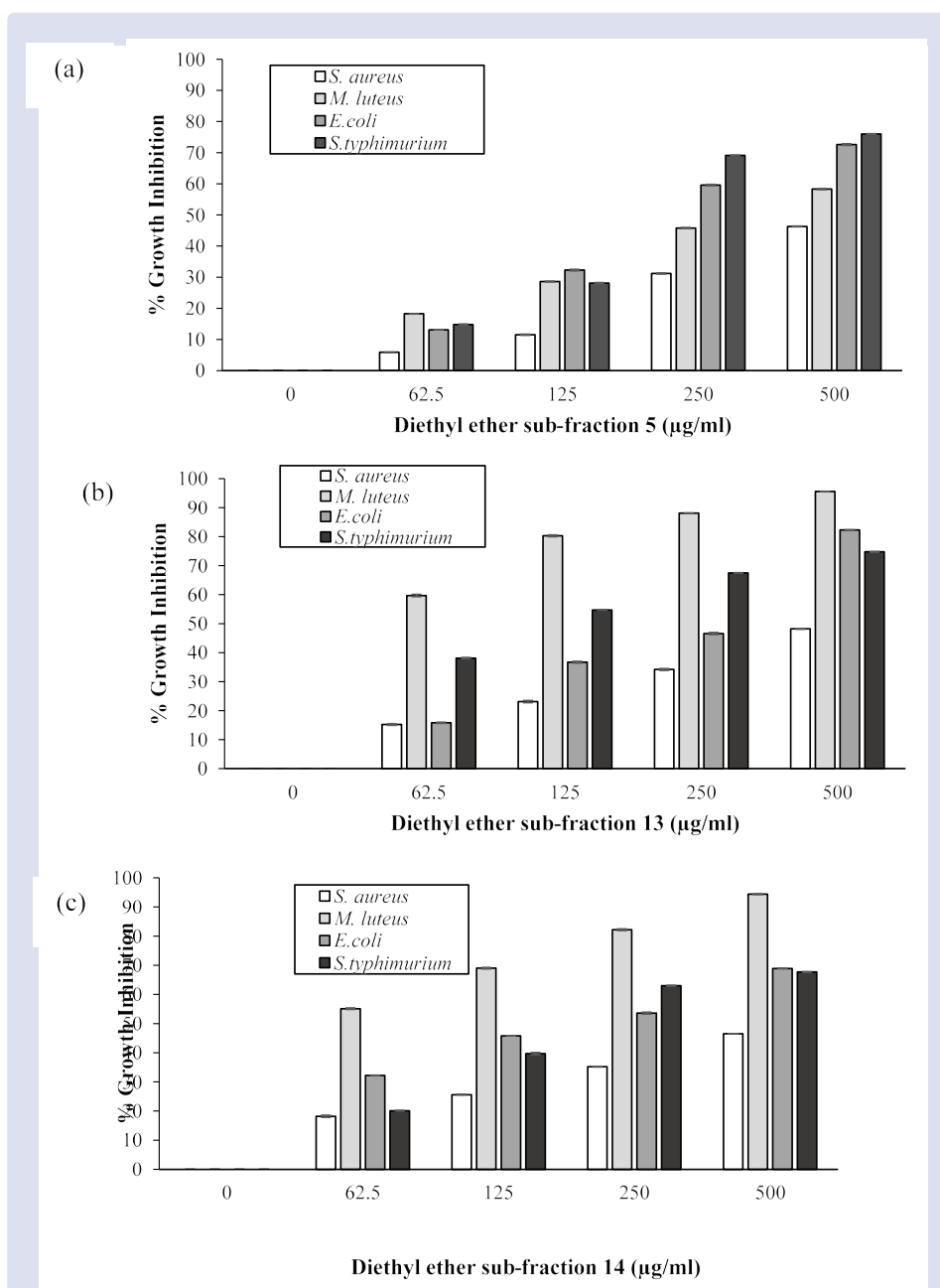


Figure 2: Antibacterial activity of diethyl ether sub-fraction 5 (a), sub-fraction 13 (b) and sub-fraction 14 (c) of *X. exigua* at various concentrations using broth microdilution method challenged by four different bacterial test strains (*S. aureus*, *M. luteus*, *E. coli*, and *S. typhimurium*). Percentage of growth inhibition was expressed as mean \pm S.D.

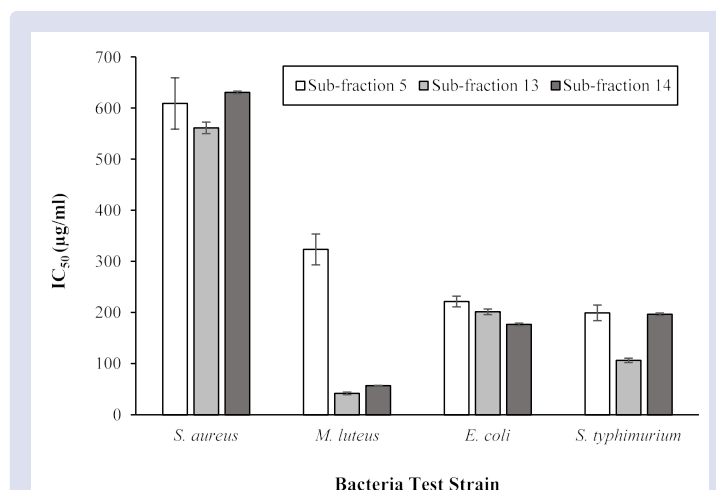


Figure 3: Half minimal inhibitory concentration (IC_{50}) values (mean \pm S.D.) of diethyl ether sub-fraction 5, sub-fraction 13 and sub-fraction 14 of *X. exigua* against five ATCC bacteria test strains (*S. aureus*, *M. luteus*, *E. coli* and *S. typhimurium*).

recorded by the lowest IC_{50} value at 176.7 ± 2.4 (Figure 3). In contrast, the diethyl ether of SF 5 produced the lowest potency against *M. luteus* ($IC_{50} = 323.4\pm 30.2$), *E. coli* ($IC_{50} = 221.5\pm 10.5$), and *S. typhimurium* ($IC_{50} = 199.3\pm 15.2$) than the diethyl ether of SF 13 and SF 14.

DISCUSSION

Marine sponges are known to possess many bioactive compounds, which are of critical importance for the development of new antibacterial drugs.²⁰⁻²⁵ In this study, we screened antibacterial activity from methanolic extracts of three samples of marine sponges (*X. exigua*, *X. muta*, and *I. baculifera*). Results showed that only the extracts of *X. exigua* gave a moderate activity towards *S. aureus* and *E. coli* tested but not towards *X. muta* and *I. baculifera*. Both samples exhibited weak activity against *M. luteus*. The preliminary screening indicated that *X. exigua* has a broad-specificity against Gram-negative and -positive bacteria, but narrow-specificity only to Gram-positive bacteria for *X. muta* and *I. baculifera*. According to Qaralleh *et al.*,²⁶ differences in the chemical concentration and composition among the species might be the contributing factors to the full range variation of the antibacterial activity. In their findings, Qaralleh *et al.*²⁶ found that methanol extracts of *X. exigua* gave an antibacterial activity against *S. aureus*, *Bacillus cereus*, and *E. coli* with the diameter of inhibition of 9.5, 9.5, and 7.5 mm, respectively.

In the present study, the organic extracts of methanol are hydrophobic with non-polar properties; thus, displaying antibacterial activity. Whereas the aqueous extract of water consisting of hydrophilic with polar properties displayed no antibacterial activity. Methanol is preferred as an organic solvent to extract multi-variable types of hydrophobic compounds because it can extract both non-polar and polar compounds, as well as proteins.²⁷ This finding was in agreement with Touti *et al.*²⁸ who found very weak antibacterial activity in the aqueous extract (water) from seven Tunisian marine sponges compared to the organic phase of ethyl acetate extracts. Another report by Ankisetty and Slattery²⁹ suggested that the mixture of dichloromethane/methanol (1:1 v/v) to extract *Xetospongia* sp. displayed a satisfactory result of antibacterial activity against both Gram-negative bacteria (*P. aeruginosa*) and Gram-positive bacteria (*Mycobacterium intracellulare*) with IC_{50} values of 2.07 and 1.03 $\mu\text{g/mL}$, respectively. The present study also found that the compound extracted from *X. exigua* using diethyl ether, which was classified as the non-polar solvent, gave better antibacterial activity than the polar phase of butanol extract suggesting

that the natural existence of non-polar compounds exhibiting antibacterial properties. Hence, this finding strongly refuted the theory of polar compounds displaying antibacterial properties.

Based on the GC-MS analysis, all diethyl ether sub-fractions of *X. exigua*, namely SF 5, SF 13, and SF 14 showed the abundance of bis(2-ethylhexyl) phthalate (BEP). The potent inhibitory activity against *S. typhimurium* in all diethyl ether sub-fractions 5, 13, and 14 provided suggestive evidence of the potential of BEP as an antibacterial agent in killing Gram-negative bacteria. Several scientific evidence supports the finding that BEP isolated from marine-derived sponges and microorganisms acts as a potent antimicrobial agent.³⁰⁻³³ Chemical profiling using marine-derived actinobacteria, *Nocardiopsis* sp. SCA21, as reported by Siddharth and Ravi³² found that BEP showed a broad-spectrum inhibitory activity against MRSA (ATCC NR-46171), MRSA (ATCC 46071), *K. pneumoniae* (ATCC 13883), *Bacillus subtilis* (ATCC 6633), and *S. aureus* (ATCC 12600) with MIC values in the range of 7.81–250 $\mu\text{g/mL}$. Moreover, Rajivgandhi *et al.*³³ observed BEP-treated colistin-resistant extract on *P. aeruginosa* and *K. pneumoniae* resulted in the occurrence of the irregular shape of membrane morphology and caused the death of both bacterial strains. Hence, this study can provide an insight on BEP as a biocontrol agent in the treatment of Methicillin-resistant *Staphylococcus aureus* (MRSA) that is rampantly occurring in hospital patients worldwide.

Besides their potential as an antibacterial agent, the drawback of phthalate derivatives is that they have been widely used as a source of plasticiser. The US Environmental Protection Agency (US EPA)³⁴ has listed six phthalate plasticisers as priority marine pollutants such as dimethyl phthalate (DMP), diethyl phthalate (DEP), dibutyl phthalate (DBP), benzyl butyl phthalate (BBP), diethylhexyl phthalate (DEHP), and dioctyl phthalate (DnOP). The lethal effect of phthalate derivatives has resulted in the reduction of phytoplankton biomass community, as well as causing the structural changes and decrease of Chl-a concentration at 50%.³⁵ The DEHP is the most common carcinogenic compounds that may impair neurodevelopment, respiratory, and reproductive systems upon dispersal in the environment. For instance, low birth-weight and retarded growth and development of the placenta occurred in rats treated with DEHP at 125 mg/kg/day.³⁶ Furthermore, the DEHP has been known as a potent compound to trigger a metabolic dysfunction by inhibiting the liver function, glucose absorption, aromatase activity, the expression of insulin receptor, GLUT4

proteins, growth of follicle, increasing the gonadotropin serum level at high dose (30 mg/kg be/day), and promoting lipid accumulation in hepatocytes.³⁷⁻³⁹

Hexadecanoic acid is one type of straight-chain saturated fatty acid, which is commonly found in plants and animals. According to Pu *et al.*,⁴⁰ the hexadecanoic acid and its derivatives from neem oil could inhibit the growth of *S. aureus*, *E. coli*, and *Salmonella* sp. with the MIC within the range of 20 to 0.625 mg/mL. Our study indicated that hexadecanoic acid constitutes one of the most abundant isolated compounds from *X. exigua*. Our screening results coincided with Parsons *et al.*⁴¹ who found that brown algae, *Cytosoria compressa* and marine sponge, *Spongia officinalis* extracts contained fatty acids such as hexadecanoic and octadecanoic acids as the primary component, which play a vital role as anionic surfactants under low pH targeting the disruption of bacterial cell wall and membranes.⁴²

From the previous findings, we determined that the IC₅₀ value for all diethyl ether sub-fractions of *X. exigua* suggested that the Gram-positive bacteria were more sensitive than Gram-negative bacteria. These findings were in agreement with many documented studies on the antibacterial property of sponges that showed Gram-positive bacteria are more susceptible to marine sponges extracts as compared to Gram-negative.^{43,44} The reasons are possibly due to the biochemical composition of the outer membrane of the Gram-negative bacteria consisting of lipoprotein and lipopolysaccharide, which are selectively permeable and resistant, thereby modulates the uptake of antimicrobial compounds.⁴⁵

To the best of our knowledge, this study is believed to be the first report on the identification of bioactive compounds potentiating antibacterial properties from the sub-fraction of HPTLC of *X. exigua*. One of the limitations of this study is that the antibacterial activity of the *X. exigua* was performed on only four pathogens. Therefore, future study is recommended to expand more pathogens with a particular focus on life-threatening diseases such as HIV, malaria, and endemic cases of MRSA.

CONCLUSION

Three sub-fractions of diethyl ether (SF 5, SF 13, and SF 14) of *X. exigua* actively exhibited antibacterial activity. SF 5 consisted of five compounds, namely 1-eicosanol, hexadecanoic acid butyl ester, octadecanoic acid butyl ester, eicosanoic acid-2-hydroxyethyl ester, and bis(2-ethylhexyl) phthalate, while SF 13 consisted of four compounds, namely n-hexadecanoic acid, octadecanoic acid butyl ester, bis(2-ethylhexyl)phthalate and 2-tert-butyl-4,6-bis(3,5-di-tert-butyl-4-hydroxybenzyl)phenol. The final sub-fraction, SF 14, consisted of three compounds, namely hexanedioic acid, bis(2-ethylhexyl)ester, bis(2-ethylhexyl) phthalate, and 9-19-cycloplanostan-3-ol-acetate (3 β). SF 13 and SF 14 could inhibit the growth of all bacteria tested, indicating a broad-spectrum activity, but SF 5 showed selective inhibition only towards *E. coli* and *S. typhimurium*.

CONFLICTS OF INTEREST

None.

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ABBREVIATIONS

BEP: bis(2-ethylhexyl) phthalate, BMM: Broth Microdilution Method, BUF: Butanol fraction, DDT: Disc Diffusion Test, DEF: Diethyl ether

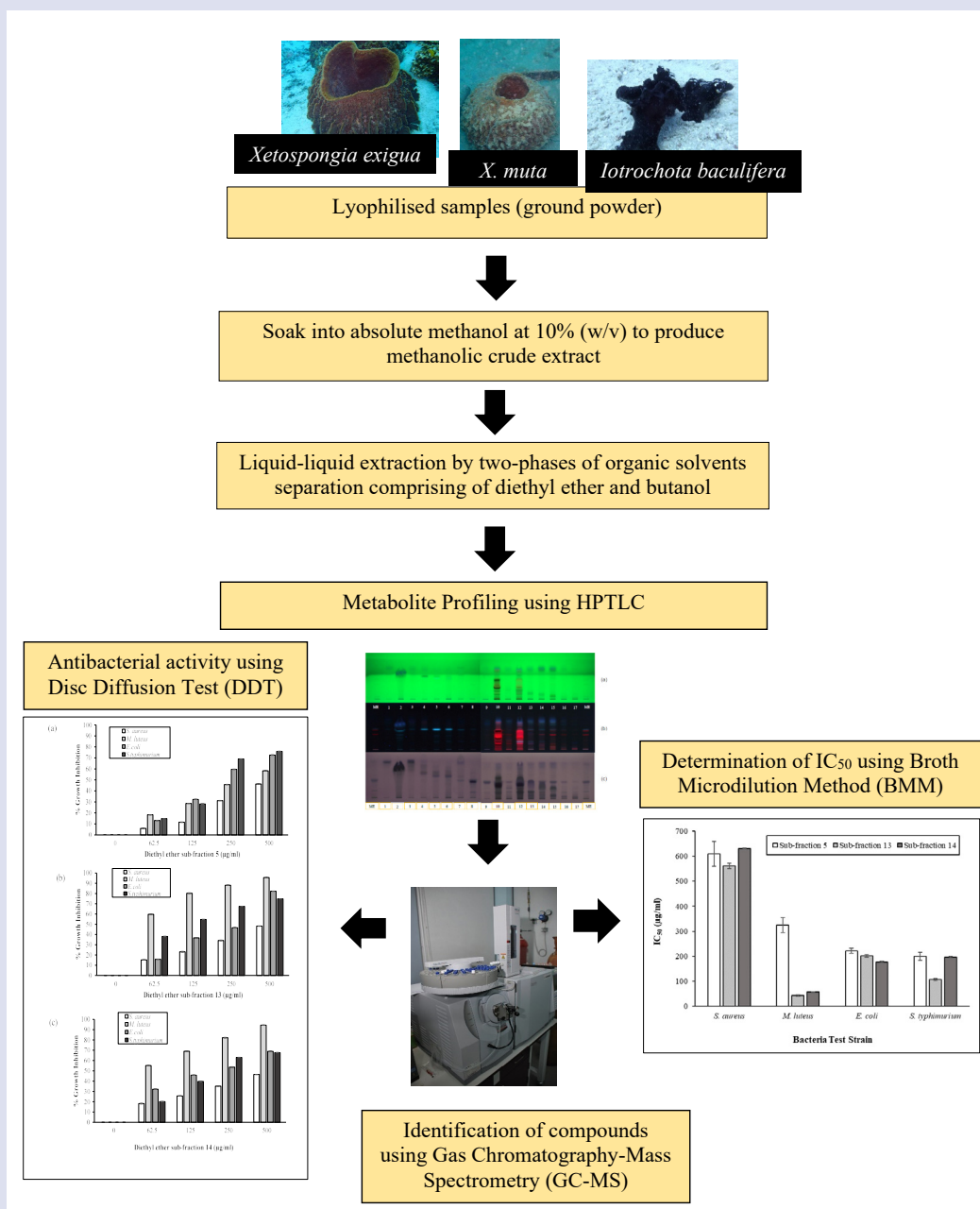
fraction, GC-MS: Gas Chromatography-Mass Spectrometry, IC₅₀: Half Maximal Inhibitory Concentration, GMD: Golm Metabolome Database, HPTLC: High-Performance Thin Layer Chromatography, MHB: Mueller Hinton broth, MIC: Minimum Inhibitory Concentration, MRSA: Methicillin Resistant *Staphylococcus aureus*, NIST: National of Standards and Technology.

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GRAPHICAL ABSTRACT



SUMMARY

- In this study, the antibacterial potential of three selected marine sponges (*Xetospongia exigua*, *X. muta*, and *I. baculifera*) was evaluated using DDT and BMM methods.
- The bioactive constituents of the marine sponges were profiled and identified using HPTLC and GC-MS.
- Out of the three marine sponges samples studied, only the diethyl ether fractions of *X. exigua* possessed a strong antibacterial response, but no activity towards methanol fractions.
- SF 13 and SF 14 of *X. exigua* could inhibit the growth of all bacteria tested, indicating a broad-spectrum activity. However, SF 5 showed selective inhibition only towards *E. coli* and *S. typhimurium*, indicating a narrow-spectrum activity.

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Noraznawati Ismail, Ph.D. Associate professor at the Institute of Marine Biotechnology, Universiti Malaysia Terengganu. Currently, she is head of marine microbes' research division. Her recent research interest is on marine biotechnology focuses on the discovery of potential bioactive metabolites in marine organisms particularly in marine microbes, sponges and horseshoe crabs. This includes exploring for potential microbes associated with marine organisms and functional protein from haemolymph plasma of horseshoe crabs particularly involve in coagulation factors.

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