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Review

BIOMEDICAL POTENTIALS OF INTERTIDAL MARINE ORGANISMS FROM SINGAPORE

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ABSTRACT

As part of a pilot scale project on drug discovery from local marine organisms, 19 intertidal marine organisms from Singapore waters were collected and screened for the presence of biologically-active natural products. These marine organisms were collected due to the ease of procurement and their relative abundance. The organic extracts of these organisms were prepared and screened in the brine shrimp lethality (BSL), the cytotoxicity (MCF-7 and MOLT-4 cell lines), and the quorum sensing inhibition (QSI) assays. Over 60% of the extracts gave significant biological activities in the BSL and the cytotoxicity (MOLT-4) assays when tested at 1000 ppm. Three sponge extracts showed moderate antibacterial activity while a fraction obtained from the gross fractionation of the extract of the marine cyanobacterium, Lyngbya majuscula (PH2), exhibited anti quorum sensing activity in the QSI assay. Lyngbya majuscula (PH2) also exhibited exceptional biological properties in the toxicity assays and its extract underwent further fractionation. The ^{1}H-NMR spectra of the bioactive chromatographic fractions derived from the microalgal extract indicated the presence of unique lipopeptides. Data from this study provided rationale to initiate marine natural products research for drug discovery in Singapore.

Key words: drug discovery, cytotoxicity, brine shrimp lethality assay, anti-quorum sensing

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INTRODUCTION

Secondary metabolites from marine organisms are an important source of biomolecules for drug discovery and development (Newman and Cragg, 2004). Since its inception in the 1950s, a myriad of novel marine-derived compounds, with unique carbon skeleton

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never been reported from terrestrial source, have been identified (Blunt et al., 2007). A number of these natural products possess potent biological properties and currently are either in preclinical or clinical testing for the treatment of various human ailments (Newman and Cragg, 2004). These include anticancer agents such as bryostatin A from the bryozoan *Bugula neritina* (Kortmanski and Schwartz, 2003), ecteinascidin 743 (ET-743) from the colonial tunicate *Ecteinascidia turbinata* (Fayette et al., 2005), and discodermolide from the sponge *Discodermia dissoluta* (De Souza, 2004); the anti-inflammatory agent, pseudopterosin A from the gorgonian *Pseudopterogorgia elisabethae* (Mayer et al., 1998), and the anti-malarial agent, manzamine A from the bacterium *Micromonospora* sp. (Ang et al., 2001). The emergence of multi-resistant strains of pathogenic bacteria, hard to treat diseases as well as the threat of new epidemics, such as the bird flu virus, drive the need to develop new therapeutics, and scientists are turning to the these marine organisms for new drugs.

An important component that contributes to the success of marine natural products research program is the ease of access to biodiversity (Higa et al., 2001; Berlinck et al., 2004; Lee and Chang, 2004). In spite of large scale coastal development and reclamation works, the waters surrounding the island of Singapore support a wide diversity of marine organisms. A majority of these marine organisms are dwellers of coral reefs located off the southern coast of Singapore. For instance, a variety of marine invertebrates and algae have been recorded from local waters, including at least 20 species of nudibranchs, 31 species of gorgonians, more than 50 sponge species as well as a diverse algae flora (Chou, 1992; Goh and Chou, 1996; Sachidhanandam et al., 2000; Lim and Tan, personal observation). Furthermore a number of sponge genera (e.g. *Mycale* sp., *Halichondria* sp., and *Haliclona* sp.) known to produce potent cytotoxic molecules currently in preclinical testing as anticancer agents in the North America (Newman and Cragg, 2004) are also found in Singapore. Due to the diversity of marine life in Singapore, a pilot study was initiated to screen local marine organisms for the presence of bioactive compounds.

In the present study, a variety of easily accessed marine organisms were collected from three intertidal locations (St. John's Island, Pulau Hantu, and Raffles Marina) in Singapore to screen for bioactive natural products. Their organic extracts were prepared and screened in three in-house bioassay systems, including the brine shrimp (*Artemia salina*) lethality (BSL) assay, the cytotoxicity assay based on the human breast carcinoma cancer (MCF-7) and T lymphoblastic leukemia (MOLT-4) cell lines, and the quorum sensing inhibition (QSI) assay using the mutant biosensor strain *Chromobacterium violaceum* CV026. The brine shrimp lethality assay is a quick and easy method to establish toxicity of extracts and is therefore chosen as one of the bioassays for this reason. Furthermore, studies have shown good correlation between the BSL and the cytotoxicity assays (Carballo et al., 2002). Organic extracts active in these assays could yield natural products as potential anticancer agents.

The anti-pathogenic activities of the extracts were also explored using the quorum sensing inhibition assay. The mutant strain *Chromobacterium violaceum* CV026 was used as a biosensor to detect anti-pathogenic activity (McClean et al., 1997; McLean et al., 2004). It has been reported that many gram-negative bacteria, including pathogenic species (e.g. *Pseudomonas aeruginosa*), use small organic molecules known as *N*-acylated homoserine lactones (AHLs) for cell-cell communication. When a bacterial colony reaches a certain cell density, the resulting high concentrations of AHLs regulate the

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expression of many bacterial genes, including bioluminescence (Fuqua et al., 1996), antibiotic biosynthesis (Wood and Pierson, 1996), and cytotoxins production (Winson et al., 1995), through interaction with a transcriptional activator protein (Waters and Bassler, 2005). Such cell density-dependent phenomenon is termed as bacterial quorum sensing and recent studies have shown that the interference of such a system represents a novel way of controlling bacterial pathogeneses (Raffa et al., 2005; Rasmussen and Givskov, 2006).

MATERIALS AND METHODS

Collection of intertidal marine organisms.

Samples of 19 marine organisms were collected by hand from three locations in Singapore during low tide at St. John's Island (SJ, 1°13'10.92″N, 103°50'51.83″E), Raffles Marina (RM, 1°20'34.92″N, 103°38'04.25″E), and Pulau Hantu (PH, 1°13'31.44″N, 103°44'55.89″). Identification of marine organisms was carried out by SCL, KST, and TLT and listed in Table 1. Voucher samples of each species were set aside and stored at -80°C at the National Institute of Education (NIE).

Extraction of intertidal marine organisms.

Each collected specimen was rinsed with distilled water to remove accompanying debris and finely chopped before seeping in methanol and left standing for 24 h. The methanol extracts of each specimen were filtered and the solvent removed under vacuum using a rotary evaporator. The resulting aqueous extracts were subjected to solvent partitioning to remove the organic component using chloroform. Upon removal of the solvent in vacuo, the dried organic extracts were reconstituted in small amounts of EtOAc and transferred to 8 dram vials for storage at -30 °C.

Vacuum flash chromatography (VFC) of organic extract from Lyngbya majuscula (PH2).

The organic extract (ca. 500 mg) of *Lyngbya majuscula* (PH2) collected from Pulau Hantu were subjected through vacuum flash chromatography (VFC) on normal phase Si using a combination of hexanes, EtOAc, and MeOH of increasing polarity. Eight fractions were obtained and solvents were removed in vacuo using a rotary evaporator before storage in 4 dram vials in CH_2Cl_2. All fractions were assayed at 1 and 10 ppm in the brine shrimp lethality assay and tested at 2.0 mg in the quorum sensing inhibition assay using the agar well method described below.

Proton NMR analysis.

Proton-NMR spectra of fractions 4, 5 and 6 obtained from VFC of the organic extract of *Lyngbya majuscula* (PH2) were recorded on a 400 MHz Bruker NMR spectrometer with the solvent $CDCl_3$ used as an internal standard (δ_H at 7.26).

Brine shrimp (Artemia salina) lethality (BSL) assay.

Each organic extract was tested at 1000, 100, and 10 ppm in the brine shrimp (*Artemia salina*) lethality assay following the protocol of Carballo et al. (2002). Each concentration

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was performed in duplicate. Percentage mortality was determined after 24 h and calculated as number of dead brine shrimp larvae/total number of brine shrimp larvae × 100%. Controls contained only artificial seawater.

Cytotoxicity assays.

Cytotoxicity was measured in MOLT-4 and MCF-7. MOLT-4 is a human T lymphoblastic leukemia cell line and MCF-7 is a human breast carcinoma cell line. MOLT-4 cells were cultured in RPMI-1640 medium while MCF-7 cells were cultured in MEM medium and were allowed to become confluent prior to every treatment. Cell viability was determined by trypan blue exclusion. Cells were diluted in 0.9% NaCl, followed by 0.1% trypan blue dye. The appearances of dead cells were stained blue. Cell viability and cell density (number of cells/ml) were then determined microscopically using a hemacytometer. Cytotoxicity of the extracts was determined using the MTT (3[4,5-dimethylthiazol-2-yl]2,5-diphenyltetrazoliumbromide) assay. Specific volumes of medium, cells, and extract were added into the respective wells of a 96-well microplate under sterile conditions.

a. Preparation of extracts.

From each organic extract, 8.0 mg was weighted out for use in the cytotoxicity assay. These extracts were then separately dissolved in 1 ml of 10% DMSO solution to obtain a concentration of 1000 ppm. Serial dilution was carried out to obtain concentrations of 100 ppm and 10 ppm.

b. MOLT-4 cell line.

Each test extract concentration was set up by re-suspending 40,000 cells in 70 µl of RPMI 1640 cell culture medium supplemented with 10% foetal bovine serum (FBS). A total of three concentrations of the extracts were prepared by dissolving the original extract in DMSO, which were then added in 10 µl portions to the cells such that the final concentrations of the extracts tested were 10, 100, and 1000 ppm. The final concentration of DMSO in all the extracts to be tested was 1.25% (v/v). Following an incubation of the cells with the different concentrations of the extracts for 24 h at 37^{o}C and 5% CO_2, 20 µl of a 5 mg/ml solution of MTT was added to all the test extracts (final MTT concentration at 1 mg/ml). The test extracts were incubated for another 3 h after which a 100 µl of lysing buffer solution (20% sodium dodecyl sulfate dissolved in 50% DMF, pH adjusted to 4.7 with acetic acid) was added and left overnight. The absorbance was then read at 570 nm against the standard mixture of RPMI 1640 medium, MTT, and lysing solution as blank.

c. MCF-7 cell line.

The procedures were similar to MOLT-4 cells. The only difference was that only 20,000 cells were used for each test extract concentration. The cells were re-suspended in 70 µl of MEM cell culture medium supplemented with 10% FBS and were allowed to become confluent prior to treatment with the extracts.

Quorum sensing inhibition (QSI) assay.

The biomonitor strain, *Chromobacterium violaceum* CV026, was used in the QSI assay. This mutant strain is unable to produce the signaling molecule, C-6-acyl homoserine

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lactone (C-6-AHL), but responds to an exogenous source leading to the production of a purple pigment, violacein, when bacterial cells reaches a certain cell density (McClean et al., 1997). The production of violacein in the wild type strain is regulated by quorum sensing. Two methods were used in the QSI assay, the disk diffusion and the agar well diffusion methods.

a. Disk diffusion method.

Each organic extract (tested at 5.0 mg and 0.5 mg in duplicate each) was loaded onto a sterile 10 mm filter paper disk and placed onto prepared LB plates inoculated with overnight culture (100 µl) of *Chromobacterium violaceum* CV026 and 100 µl (5 µM) of the autoinducer, C-6-acyl homoserine lactone (C-6-AHL). Each organic extract was reconstituted in small amounts of chloroform before transferring onto a filter paper disk and left to dry completely before placing them onto the agar. Only chloroform was transferred to the control disks. All agar plates were then incubated overnight at 30^{o}C. Anti-quorum sensing activities can be detected by a ring of colorless but viable cells around the disk. Measurements were made from the outer edge of the disks to the edge of the zones of anti-QS inhibition.

b. Agar well diffusion method.

Five wells of 10 mm in diameter each were made in each LB agar plate [preinoculated with culture (100 µl) of *Chromobacterium violaceum* CV026 and 100 µl (5 µM) of C-6-AHL] using a sterile cork-borer. Each organic extract was tested at 5.0 mg and 0.5 mg in duplicate. The extracts were reconstituted in 100 µl of LB broth before transferring into wells. Each plate contained a control well with only 100 µl of LB broth. All treated agar plates were incubated at 30^{o}C overnight. Measurements of anti-quorum sensing activities were made from the outer edge of the wells to the edge of the zones of inhibition.

RESULTS AND DISCUSSION

Results

Brine shrimp lethality and cytotoxicity assays.

A majority of the marine-derived extracts (about 60% or 12 species), with the exception of *Ceratodictyon spongiosum* (PH3), *Dactylospongia* sp.3 (PH4), *Spheciospongia* sp.3 (PH5), and *Haliclona* sp.1 (RM2), exhibited significant activity (> 80% mortality) at the highest concentration of 1000 ppm (Table 1). Furthermore, extracts of three marine species, *Dysidea* sp. (SJ9), *Symplegma* sp. (RM1), and *Lyngbya majuscula* (PH2), showed consistently high biological activity (>50% mortality) at all test concentrations. For instance, the organic extract of *Dysidea* sp. (SJ9) exhibited 100% mortality rates for all test concentrations while that of *Lyngbya majuscula* (PH2), gave mortality rates at 80%, 95%, and 100% at 10, 100, and 1000 ppm, respectively **(Table 1).**

In the cytotoxicity assay, about 70% of the marine extracts (from 14 species) showed high biological activity (> 80% inhibition) when tested at 1000 ppm against the MOLT-4 cell line. However, only 36% (seven species) of the organic extracts showed greater than 80% inhibition when tested at 1000 ppm against the MCF-7 cell line (Table 1). When all three concentrations were considered, only extracts of four species [*Spheciospongia* sp.3 (PH5), *Haliclona* sp.2 (RM3), *Synaptula* sp. (RM5), and *Lyngbya majuscula* (PH2)] and three species

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[*Spheciospongia* sp.3 (PH5), *Synaptula* sp. (RM5), and *Lyngbya majuscula* (PH2)] indicated significant activity (> 50% inhibition) in the MOLT-4 and MCF-7 cell lines, respectively. Of the 19 marine species, only the organic extracts of *L. majuscula* (PH2) showed consistently high activity (> 50% toxicity) at all test concentrations in both the BSL and the cytotoxicity (MOLT-4 and MCF-7 cell lines) assays.

Quorum sensing inhibition (QSI) assay.

In the QSI assay, the inhibition of the purple pigment, violacein, production in the mutant strain *Chromobacterium violaceum* CV026 by any marine extracts is an indication of quorum sensing (QS) interference. However, in the present study none of the marine-derived extracts showed QS inhibition in both the disk and agar well diffusion methods. Instead, three marine extracts of *Dactylospongia* sp.1 (SJ4), *Dactylospongia* sp.2 (SJ8), and *Dysidea* sp (SJ9) showed antibiotic activity (Table 2). Antibiotic activities in these extracts were indicated by a clear or transparent zone of inhibition as compared to true QS inhibition where there should be a confluent layer of bacteria with no violacein production (McLean et al., 2004). True QS inhibition was observed for one of the fractions (fraction 3) obtained from vacuum flash chromatography of the organic extract from *Lyngbya majuscula* (PH2) **(Figure 1 and Table 2).**

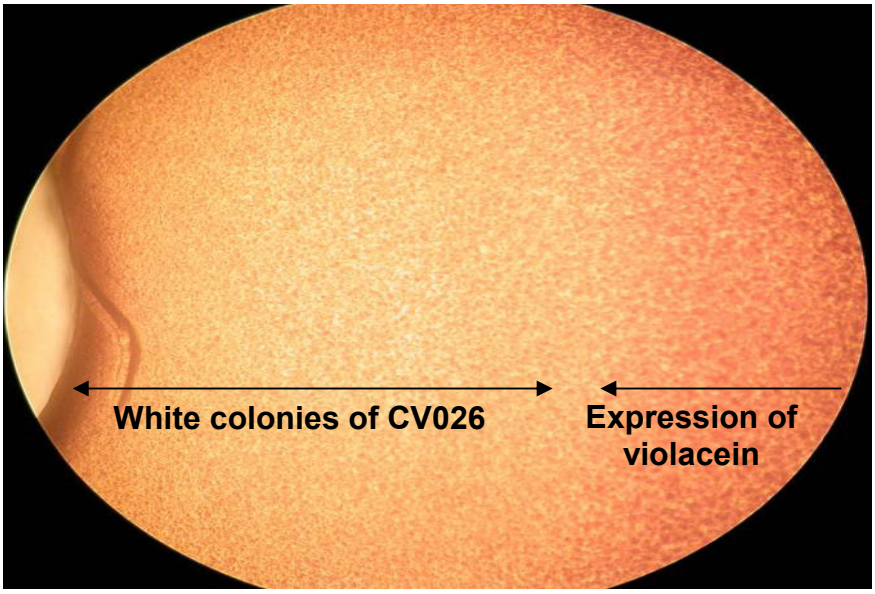

Fig 1. Magnification of the area around a well containing the bioactive fraction 3 obtained from VFC of the organic extract of *Lyngbya majuscula* (PH2). There is a confluent layer of bacteria with no production of violacein (anti-quorum sensing effect) in proximity to the well.

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Table 1. Toxicity of Marine-Derived Organic Extracts in the Brine Shrimp Lethality (BSL) and Cytotoxicity Assays Based on the MCF-7 and MOLT-4 Cell lines

Organisms	Code[1]	BSL[2]			MCF-7[3]			MOLT-4[3]		
		10ppm	100ppm	1000ppm	10ppm	100ppm	1000ppm	10ppm	100ppm	1000ppm
Sponges										
Haliclona sp.1	RM2	0	0	50	25	58	83	49	81	100
Haliclona sp.2	RM3	20	55	85	26	23	56	65	92	100
Mycale sp.	SJ1	5	15	-	0	3	43	0	0	100
Spongia sp.	SJ2	45	50	85	0	3	15	4	10	99
Spheciospongia sp.1	SJ3	5	20	100	-	-	-	-	-	-
Dactylospongia sp.1	SJ4	5	40	100	0	18	40	33	32	99
Halichondria sp.	SJ5	5	65	-	0	1	11	0	0	83
Spongocladia sp.	SJ6	0	65	-	0	0	5	13	32	97
Spheciospongia sp.2	SJ7	0	35	100	0	0	0	15	24	99
Dactylospongia sp.2	SJ8	10	35	100	0	6	100	14	15	100
Dysidea sp.	SJ9	100	100	100	0	20	84	24	83	96
Ceratodicyton spongiosum	PH3	0	0	19	43	40	58	24	32	50
Dactylospongia sp.3	PH4	0	0	0	28	35	88	0	40	55
Spheciospongia sp.3	PH5	0	0	0	64	62	64	56	80	85
Tunicate										
Symplegma sp.	RM1	70	80	100	0	0	0	0	0	32
Soft Coral										
Dendronephthya sp.	RM4	0	45	100	0	6	51	13	66	94
Synaptid										
Synaptula sp.	RM5	15	80	100	50	100	97	100	100	100
Cyanobacteria										
Lyngbya sp.	PH1	46	52	100	37	53	98	21	55	77
Lyngbya majuscula	PH2	80	95	100	66	83	90	58	84	92

[1]First two letters denotes the initials of collection site. (RM = Raffles Marina, SJ = St. John's Island, PH = Pulau Hantu)
[2]Data is expressed as average % mortality in the brine shrimp lethality assay.
[3]Data is expressed as % inhibition in the cytotoxicity assays.

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Table 2. Antimicrobial and Anti-Quorum Sensing Activities of Marine-Derived Test Samples in the QSI Assay Measured as Zone of Inhibition in mm

Test samples	Code[1]	Agar well method			Paper disc method	
		0.5 mg	2.0 mg	5.0 mg	0.5 mg	5.0 mg
Dactylospongia sp.1	SJ4	8	-	8	4	5
Dactylospongia sp.2	SJ8	4	-	4	5	5
Dysidea sp.	SJ9	1	-	2	0	0
Fraction 3[2]	PH2	-	4[3]	-	-	-

[1]First two letters denotes the initials of collection site. (RM = Raffles Marina, SJ = St. John's Island, PH = Pulau Hantu)
[2]Fraction 3 obtained from VFC of the organic extract from *Lyngbya majuscula* (PH2).
[3]Anti-quorum sensing activity.

Chemical analysis of bioactive fractions from the extract of Lyngbya majuscula (PH2). Due to the exceptional biological activity of the organic extract (Table 1) from *Lyngbya majuscula* (PH2) in both the brine shrimp lethality and cytotoxicity assays, it was subjected to further gross separation using vacuum flash chromatography (VFC) on normal phase Si gel. Of the eight fractions obtained from VFC, only fractions 5 and 6 showed biological activity in the brine shrimp lethality assay tested at 1 and 10 ppm (data not shown). The ^{1}H-NMR spectra of these fractions were measured in $CDCl_3$ and they indicated the presence of biomolecules belonging to either the nonribosomal polypeptide (NRP) or hybrid NRP-polyketide structural class (only ^{1}H-NMR spectrum of fraction 5 is shown in **Fig 2**). For instance, proton signals resonating between 3.50 and 5.60 ppm could be due to α-Hs' of amino acids. Low field proton signals between 6.00 and 8.00 ppm were attributable to aromatic, olefinic, and *N*H protons. In addition, singlet proton signals between 2.50 and 3.20 ppm and doublet proton signals between 1.00 and 1.50 ppm could be due to *N*-methylated protons and methyl protons of aliphatic amino acids (e.g. valine and isoleucine), respectively.

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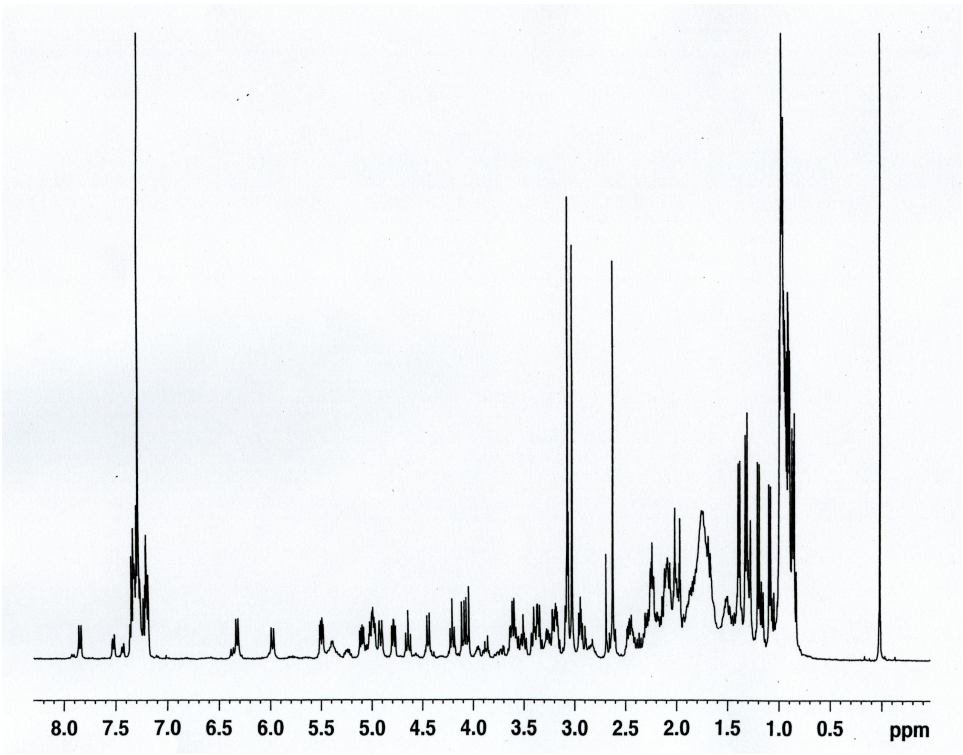

Fig 2. ^{1}H-NMR spectrum (in $CDCl_3$) of fraction 5 obtained from normal phase Si vacuum flash chromatography of organic extract from *Lyngbya majuscula* (PH2).

Discussion

In the present study, the screening of organic extracts prepared from 19 marine organisms indicated high incidence of biological activities, especially in the brine shrimp lethality and cytotoxicity assays. The brine shrimp lethality assay has been in used widely in the past 30 years to detect toxicity (Sorgeloos et al., 1978; Persoone and Wells, 1987) as well as in detecting antitumoral natural compounds (Solis et al., 1993). This assay has been instrumental in uncovering a number of important natural products [e.g. curacin A (Gerwick et al., 1994)] currently in preclinical testing as anticancer agents. The present study demonstrated that the BSL assay is a quick and efficient method in detecting biological activity in marine organisms. Coupled with data from the cytotoxicity assays, a number of extracts [e.g. *L. majuscula* (PH2) and *Dysidea* sp. (SJ9)] with exceptional biological activities have been identified from this study. In addition, preliminary chemical analysis based on ^{1}H-NMR spectra of the bioactive fractions from the VLC of the organic extract of *L. majuscula* (PH2) revealed unique polyketide-polypeptide type molecules.

A search of the literature indicated that numerous natural products having potent biological activities have been reported from this pan-tropical microorganism (for review see Gerwick et al., 2001 and Tan 2007). Majority are nitrogen-containing molecules biosynthesized by multi-modular enzymatic systems belonging to either the non-ribosomal polypeptide synthetase (NRPS) or mixed PKS (polyketide synthase)-NRPS. The bioactive fractions are currently under investigation and it is anticipated that novel secondary metabolites will be uncovered from this local strain of *L. majuscula* (PH2).

The only true anti-quorum sensing activity was discovered serendipitously in

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fraction 3 obtained from VFC of the organic extract from *Lyngbya majuscula* (PH2) (Table 2). In true anti-quorum sensing activity, a white zone of antagonism should be opaque and not transparent (Figure 1). The lack of anti-quorum sensing activity in the crude organic extracts from *L. majuscula* (PH2) could be due to bioactive molecule(s) being masked by other substances or the concentration of the active molecules were low. This is the first report of anti-quorum sensing activity detected from a marine cyanobacterial test sample. The use of *C. violaceum* CV026 is a reliable bioassay for the detection of anti-quorum sensing property in the present study. To date, three other studies have reported the use of *C. violaceum* CV026 to screen extracts from natural sources, such as medicinal plants, dietary fruits, herbs, and spices, for such activity (Adonizio et al., 2006; Choo et al., 2006; Vattem et al., 2007).

In spite of the rich biodiversity of marine organisms found in local waters, marine natural products research is currently not actively pursued in Singapore. As evident from the present study, local marine organisms represent an important source of biomedically useful natural products. Few attempts have been made to screen certain groups of local marine invertebrates, such as gorgonians, for biological activities but no chemistry has been reported from these early studies (Goh et al., 1995; Goh and Chou, 1998; Koh et al., 2000; Koh et al., 2002). There are past reports of secondary metabolites isolated from marine organisms conducted by foreign researchers (e.g. Dr. Pettit's group at the University of Arizona) or local natural products research programs (e.g. Center of Natural Products Research at the Institute of Molecular and Cell Biology) (McDonald and Ireland, 1992; Pettit et al., 1996; Nilar et al., 2002; Pettit et al., 2004). However, none of these past studies target shallow water species. Unfortunately, these programs have since been terminated. Significant biological data obtained from the present study therefore provided rationale for initiating marine natural products research as part of a drug discovery and development program in Singapore.

Conclusion

The current study is first of its kind in screening local shallow water marine species for bioactive natural products. In spite of the small sample size of 19 intertidal marine species used in the study, high incidence of biological activity were observed, especially in the brine shrimp lethality and cytotoxicity assays. One particular organic extract from *Lyngbya majuscula* (PH2) gave significant biological activity in both the brine shrimp lethality and cytotoxicity assays, providing strong impetus for further isolation work to purify the bioactive constituent(s). Three species [*Dysidea* sp. (SJ9), *Dactylospongia* sp.1 (SJ4), and sp.2 (SJ8)] showed antibacterial activity while one of the fractions from VFC of the organic extract of *Lyngbya majuscula* (PH2) indicated anti-quorum sensing activity in the QSI assay based on *Chromobacterium violaceum* CV026 biosensor. Preliminary ^{1}H-NMR spectra of the bioactive fractions obtained from the marine cyanobacterial organic extract indicated unique peptidic secondary metabolites belonging to the peptide or mixed polyketide-peptide structural class. Further purification and structure elucidation of bioactive natural products from *L. majuscula* (PH2) as well as other marine species, e.g. *Dysidea* sp. (SJ9), are currently underway and publications of these secondary metabolites will be reported in due course.

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