USES OF MARINE COMPOUNDS: EXTRACTION AND CHEMICAL PROFILING OF A CARIBBEAN MARINE SPONGE *CLATHRIA* SP.

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ABSTRACT

The purpose of this research project is to identify and chemically profile active extracts of a marine sponge, *Clathria* sp., collected from St. Thomas, US Virgin Islands. Previously, pteridine alkaloids, sterol sulfates, and bromine-containing amides collected from various species of *Clathria* have shown anti-microbial and anti-cancer activities [1]. Sponge extracts were isolated through a combination of liquid-liquid extractions, adsorption silica-gel column chromatography and high-pressure liquid chromatography (HPLC). These extracts were tested for cytotoxicity and antifouling properties through an *Artemia* sp. lethality test and

Bugula neritina, and *Balanus amphitrite* settlement biological assays. Chemical profiling was achieved through carbon and proton nuclear magnetic resonance spectroscopy (NMR) as well as low-resolution mass spectrometry. Three extracts were found to contain highly cytotoxic compounds, which should be explored further to determine their potential biomedical applications. One extract was found to contain anti-fouling properties and may hold industrial applications.

INTRODUCTION & BACKGROUND

The goal of marine natural products research is to discover and harness the therapeutic potential of compounds bio-synthesized by marine organisms and their symbiotic microorganisms. Marine sponges, tunicates, soft-coral and algae have been shown to contain a wealth of novel compounds with high anti-bacterial, anti-tumor, anti-viral, anti-inflammatory and analgesic properties [2,3]. Sponges in particular are a rich source of marine secondary metabolites due to their relative abundance, ease of collection, and the structurally diverse classes of natural products they produce [4]. Additionally, the relatively high percentage of symbiotic microorganisms in marine sponges, which can be as high as 60 percent of the total body mass, produce a varied chemical profile based upon environmental and temporal changes [5]. These secondary metabolites serve as chemical cues for larval settlement and mating and as territorial markings to prevent fouling and invasion by competitors for otherwise defenseless, soft-bodied and sessile organisms [3,6]

In 2004, forty-two marine-derived natural products were in clinical or preclinical trials [3]. Countless applications for these compounds have been identified and explored, including: the inhibition of tumor cell growth, anti-viral activity against Hepatitis B, and the treatment of Alzheimer's disease [7-10]. The mechanisms by which the compounds work are similarly diverse. Discodermolide, a polyketide isolated in 1990 from the Caribbean sponge *Discodermia dissoluta* acts to inhibit tumor cell growth [8]. Eleutherobin, first isolated from the soft coral *Eleutherobia* sp. from Western Australia is also a potent cancer-cell inhibitor [11]. Both Discodermolide and Eleutherobin share a similar microtu-

bule-stabilizing anti-mitotic mechanism with Taxol, the current leading drug for Ovarian Cancer therapy, first isolated and characterized from the tissue of the Pacific Yew tree in 1962 [11,12]. Ecteinaiscidin 743 (Trabectedin), from the Caribbean tunicate *Ecteinascidia turbinata*, is in clinical use in Europe and Korea in the treatment of soft-tissue sarcoma [13]. Additionally, Trabectedin is in multiple phase II and III clinical trials for breast, ovarian, prostate, and various sarcoma cancers [13,14]. Aside from the possibilities as therapeutic agents, marine natural products are being investigated for potential use as industrial additives to prevent biofouling.

Marine growth on man-made, underwater structures costs the shipping industry millions of dollars yearly [15]. Currently many of the anti-fouling additives in commercial use contain heavy metals, which are toxic to non-target organisms and bioaccumulate in the food chain [15,16]. Algal and invertebrate marine sources naturally and selectively inhibit fouling. An example, a dibrominated cyclopeptide from the marine sponge *Geodia barretti* was recently found to inhibit settlement of barnacle larvae [17]. With natural products such as this as templates, new alternatives to the overly toxic compounds in use today could be employed to more safely and effectively control these problem organisms.

The sponge of interest in this project, a *Clathria* species, has previously been shown to contain novel pteridine alkaloids, sterol sulfates, and bromine-containing amides, including Clathsterol, Clathrin A-C, Pseudoanchynazine A-C, Clathriol, Clathrynamide A-C, Mirabilin G, Microcionamides A and B, and Clathryimins A and B [1,18-24]. Clathsterol, a sulfated sterol, has shown activity against human immunodeficiency virus type 1 (HIV-1) by inhibiting the reverse transcriptase enzyme. Investigation of the compound Clathrynamide A has revealed its ability to inhibit growth of human myeloid leukemia cells [1].

In general, the process leading to the discovery of a new natural product for pharmaceutical application entails: 1) preliminary biological assays, 2) chemical profiling, 3) identification of biologically active components, 4) in-depth biological screening, and 5) clinical trials. This project involves the first steps in the process, namely initial chemical profiling and biological assays to determine bio-

activity.

METHODS

Collection and Identification

A sponge sample was collected from Brewer's Bay, St. Thomas, US Virgin Islands (18˚20' N 64˚55' W) by SCUBA at a depth of 10 m in May of 2006. A voucher sample (no. 0609, Figure 1) was prepared and frozen. The sponge is upright with open tubes and has a pimpled surface and a tough and elastic texture. It has a grey-orange exterior and brown-orange interior in water and is beige in methanol. One to five cm oscules are visible, distributed around the top of the sponge. Initial field taxonomy suggests the sponge is a Clathria species, however, due to the complexity and sheer number of species of marine invertebrates, exact taxonomy is difficult to confirm.

Figure 1. Sponge sample 0609.

Extraction and Isolation

The sample was transported to Cal Poly where it was thawed and partitioned following the Kupchan Isolation Scheme, Figure 2 [25]. A 1 kg sample was soaked in 100% methanol (MeOH) for a minimum of 24 h to extract the most polar compounds. The MeOH was decanted and reserved and the procedure was repeated two more times. The sample was then extracted with 100% dichloromethane (CH_2Cl_2) in the same fashion to separate the non-polar compounds.

Solvent was removed *in vacu* and the crude CH_2Cl_2 extract was partitioned with a system of hexanes and 90% MeOH/ water (H_2O). The crude MeOH fraction was partitioned between CH_2Cl_2 and H_2O. Water-soluble compounds were further separated with t-butanol (t-BuOH) and H_2O. The CH_2Cl_2 fraction was successively partitioned with a system of 90% MeOH/ H_2O and hexanes followed by a 50% MeOH/ H_2O mixture. In total, seven crude fractions were isolated: DMM, DMH, FD, FM, FH, WW, and WB.

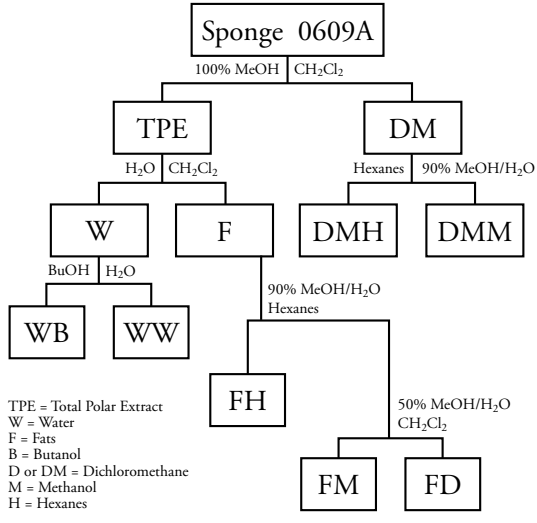

Figure 2. Kupchan Isolation Scheme

Further separation of crude extracts FD, FH, and DMH were accomplished as follows. Fractions FD and FH were combined due to similar composition according to 1H NMR data and the combined FD/FH extract was subjected to silica-gel column chromatography (eluted with a 10% v/v stepwise gradient of MeOH/ CH_2Cl_2) giving 5 fractions, FD/FH 1-5. FD/FH 4 was further separated into fractions A-F by column chromatography (eluted with a 10% stepwise gradient of EtOAc/CH_2Cl_2). DMH was separated by flash silica-gel chromatography (10% stepwise gradient of EtOAc/heptanes), yielding five fractions, DMH 1-5. Figures 3 and 4 show the detailed isolation schemes.

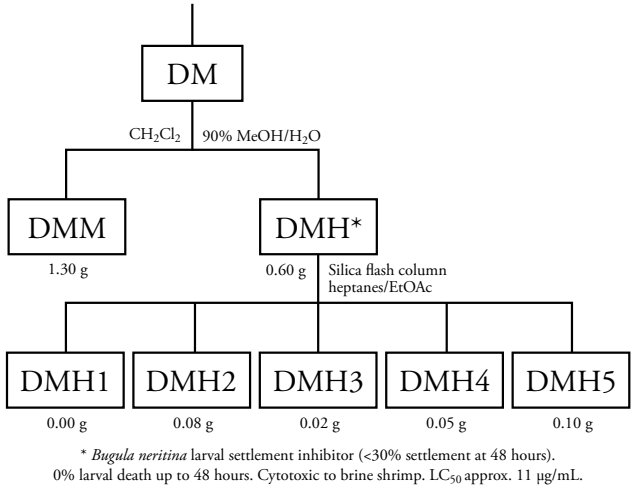

Figure 3. Isolation scheme from the crude dichloromethane (DM) extract. The weight of each fraction is shown below the respective box.

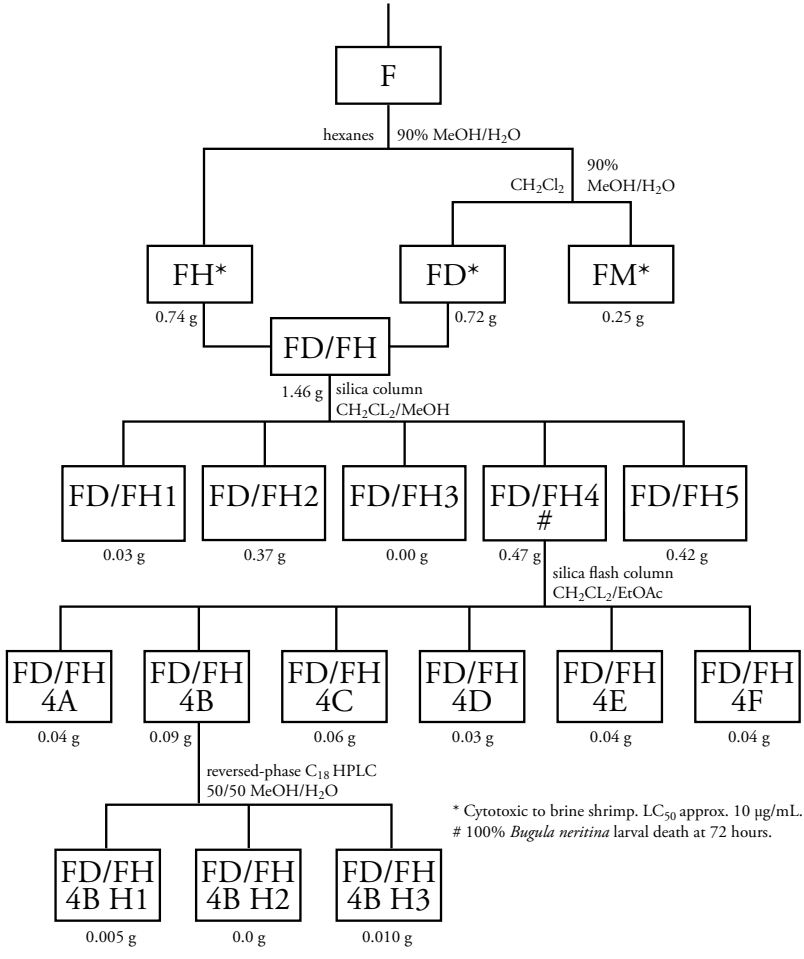

Figure 4. Isolation scheme from crude fats (F) extract.

All isolation steps were bioassay-guided, meaning that the purification of the most biologically active component was pursued. The bioassay results that dictated the isolation scheme are discussed later. NMR results also guided isolation, dependent on the presence or absence of key functional groups typical of other natural products. In some cases, such as the isolation of FD/FH 4B, sample mass dictated further purification – bioassays and complete purification of very small samples was not possible.

Bioassays

A series of bioassays were performed to determine extract cytotoxicity (*Artemia* sp. lethality test) and anti-fouling properties (*Bugula neritina* and *Balanus amphitrite* settlement bioassays). For the *Artemia* sp. bioassay, cysts were hatched in a filtered seawater solution (FSW) at room temperature in a separatory funnel. A lamp was used to provide direct light and a fish-tank pump was used for an air source. Eggs were allowed to hatch for 48 h before harvesting. Five crude extracts (FD, FH, FM, DMM, and DMH) were tested for cytotoxicity at three concentrations: 15µg/mL, 10µg/mL, and 5µg/mL in di-methyl sulfoxide (DMSO), diluted to a total volume of 5 mL with FSW. Approximately 15 live Artemia *sp.* were added to each 10 mL glass test tube. Death rate was measured at 24 and 48-hour intervals. Experimental error was corrected for with DMSO and FSW controls. Results of the bioassay are summarized in Table 1. LC_{50} values (concentration at which 50 percent death occurs) of each extract were determined by plotting the mortality rates against sample concentration (Figure 5). Fractions FD, FH, FM, and DMH all showed substantial levels of cytotoxicity. Fractions FD, FH, and DMH gave the most relevant LC_{50} values within the concentration parameters. The LC_{50} values for FD, FH, and DMH were roughly 13, 12, and 11 µg/mL, respectively.

Table 1. Percent mortality of *Artemia sp.* at 24 and 48 hour incubation times.

	% Mortality 24 Hours			% Mortality 48 Hours		
	5 µg/mL	10 µg/mL	15 µg/mL	5 µg/mL	10 µg/mL	15 µg/mL
DMM	0.0	0.0	0.0	0.0	0.0	90.9
DMH	0.0	0.0	0.0	0.0	27.3	100.0
FD	0.0	20.0	28.6	7.1	26.7	64.3
FM	0.0	50.0	81.3	66.7	57.1	100.0
FH	7.7	0.0	0.0	15.4	16.7	78.6
DMSO	0.0	0.0	0.0	0.0	0.0	0.0
FSW	0.0	0.0	0.0	0.0	0.0	0.0

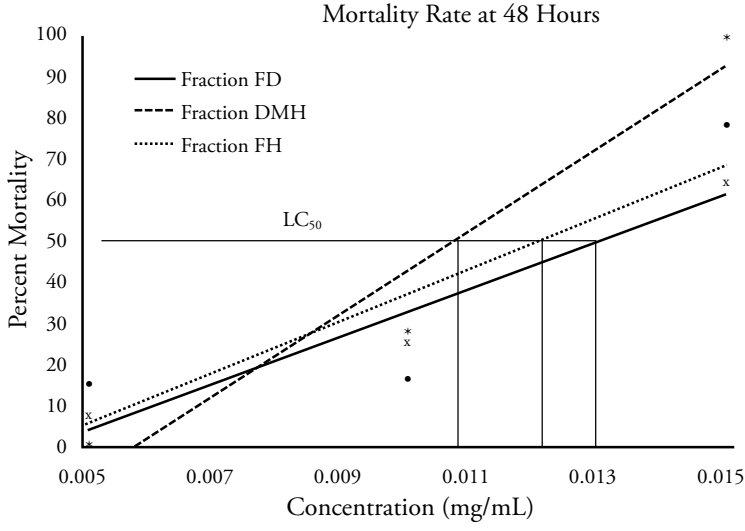

Figure 5. Mortality rate of Artemia *sp.* at varying extract concentration

A settlement bioassay was performed to determine the anti-fouling properties of the extracts. *Bugula neritina* larvae were collected from Morro Bay, CA and kept in the dark for 2 days. Larval release was induced by 2 hours of light exposure. Two-hundred µL aliquots of each extract were pipetted into a 96-well polystyrene tissue culture plate. One larva was placed into each well in a volume of 100 µl sea water, to give a final extract concentration of 5 mg/mL.The tray was incubated at 15°C and monitored for settlement and death at 3, 24, 48, and 72 hours. Fractions FM, DMM, DMH, and FD/FH 2, 4, and 5 were tested in this assay. Fractions FD and FH had been combined, due to similar cytotoxicity and NMR spectra, and further separated into fractions FD/FH 1-5. Of those, fractions FD/FH 2, 4, and 5 were chosen for the settlement assay. Bioassay results are presented in Table 2 and a graph of percent settlement and percent death after 72 hours (Figure 6) was constructed to determine the efficacy of each extract as an anti-fouling agent. The graphs reveal that of the extracts tested, DMH shows both low mortality and low larval settlement, indicating a potential antifouling

candidate. Fraction FD/FH 4, seen in Figure 6, caused 100% larvae death at 72 hours, indicating poor antifouling activity but high cytotoxicity.

Table 2. Percent settlement and death of *Bugula neritina* at 3, 24, 48 and 72 hours.

	3 hours		24 hours		48 hours		72 hours	
	% Settled	% Dead	% Settled	% Dead	% Settled	% Dead	% Settled	% Dead
DMSO	0.0	0.0	33	0.0	33	25	33	67
FSW	8.0	0.0	67	0.0	100	17	100	42
FM	25	0.0	25	0.0	100	50	100	83
DMH	25	0.0	33	0.0	33	0.0	25	25
DMM	0.0	0.0	25	0.0	42	25	42	67
FD/FH 2	33	0.0	50	0.0	58	8.0	58	50
FD/FH 4	25	0.0	25	0.0	50	42	100	100
FD/FH 5	67	0.0	75	0.0	100	8.0	67	33

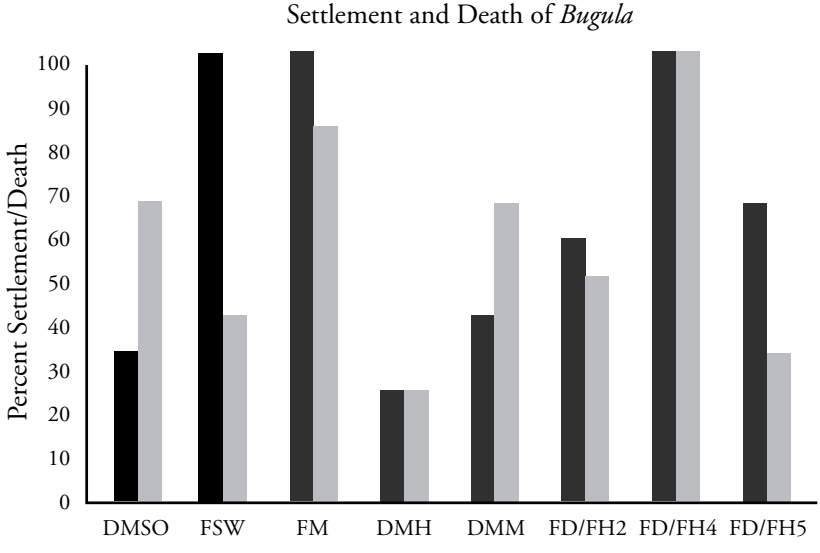

Figure 6. Percent settlement and percent death of *Bugula neritina* larvae in each of five extracts after 72 hours.

Due to promising anti-settlement activity, sub-fractions of crude extract DMH were isolated and tested. The *Bugula neritina* bioassay was unrepeatable

due to a larvae shortage, so a *Balanus amphitrite* bioassay was employed to carry out further anti-settlement tests. Barnacle cypris larvae were received from Duke University and held at 4°C. The larvae were warmed by a light source for thirty minutes prior to use to activate the cyprids. A pipette was used to transfer one larva into each well of a 96-well plate with sponge extracts previously added. Each larva was added in a volume of 100 µl, giving a final extract concentration of 1.0 mg/mL. The tray was incubated at 25°C and observed at 3, 24, 48 and 72 hours. At each observation the larvae were recorded as alive/active, dead, or metamorphosed into a juvenile barnacle. Results of the bioassay are presented in Table 3 and Figure 7. All tested fractions showed strong anti-settlement properties and Fractions DMH 2-4 also showed high cytotoxicity.

Table 3. Percent settlement and death of *Balanus amphitrite* at 3, 24, 48 and 72 hours.

	3 hours		**24 hours**		**48 hours**		**72 hours**	
	% Settled	% Dead	% Settled	% Dead	% Settled	% Dead	% Settled	% Dead
DMH	0	0	0	0	0	0	0	0
DMH 1	0	0	0	0	0	0	0	0
DMH 2	0	0	0	17	0	100	0	100
DMH 3	0	0	0	100	0	100	0	100
DMH 4	0	100	0	100	0	100	0	100
DMH 5	0	0	0	0	0	0	0	25
DMSO	0	0	8	0	17	0	33	0
FSW	0	0	50	0	58	0	58	0

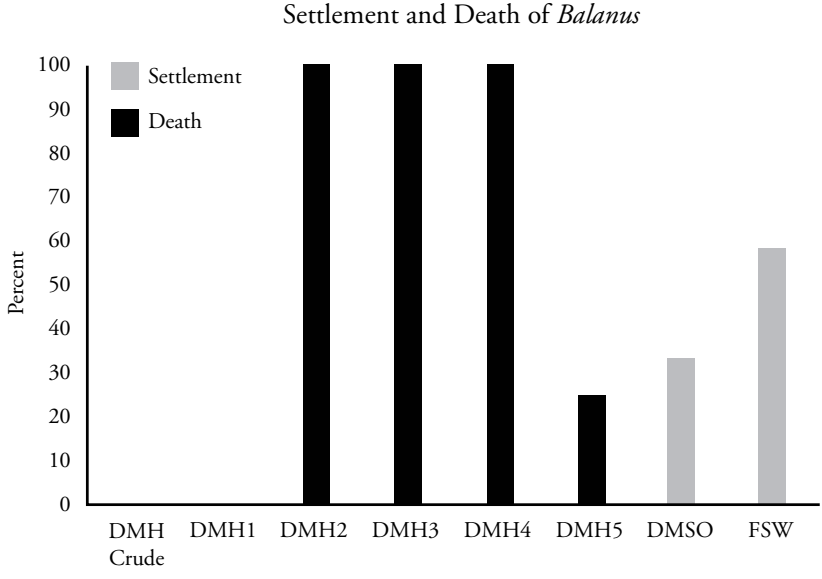

Figure 7. Percentage of *Balanus amphitrite* reached metamorphosis.

Compound Identification

To determine composition of crude fractions, molecular weights were obtained by low-resolution mass spectrometry. A summary of key chemical shifts is presented in Table 4. Molecular weight results were compared with the literature values for compounds previously isolated from *Clathria* sp. A 270.3 g/mol natural product was identified in fraction FD/FH 5 that matches closely with Clathrin B, which has a molecular weight of 270.0 g/mol. Based on molecular weight alone, the presence of Clathrin B can neither be confirmed nor rejected. It is unlikely this compound is isolatable given such a low abundance according to the mass spectra. No other molecular weight matches were found.

Table 4. Mass spectrometry data of extracts, ordered by molecular weight.

Mass (m/z)	Intensity	Time (min)	Sample
212.20	35.50	17.74	FD/FH 3
226.00	6.00	1.88	FD/FH 5
226.00	5.00	11.13	FM
253.30	5.00	3.24	DMM
264.10	14.00	1.72	DMM
264.10	19.00	1.72	FD/FH 5
270.30	24.70	12.92	FD/FH 5
278.20	110.20	19.75	FD/FH 3
278.30	10.20	19.80	FD/FH 1
278.30	13.60	19.75	FD/FH 2
294.30	13.30	11.29	FD
312.20	20.80	19.38	DMH
337.40	11.00	17.69	FD/FH 2
337.40	12.00	19.34	FD/FH 5
343.40	48.90	17.90	FD/FH 4C
384.40	23.00	19.97	FD/FH 4
398.30	1176.30	19.27	FD/FH 4C
398.30	3546.70	19.32	FD/FH 4D
451.40	114.60	18.74	FD/FH 4C
457.50	4.40	20.17	FD/FH 2
479.40	613.90	17.18	FD
479.40	405.20	17.28	FM
481.40	15.50	13.54	DMM
481.40	9.00	13.59	FD/FH 1
493.40	6.00	19.50	FD
509.50	32.10	16.80	DMH
509.50	16.30	17.90	DMH

Proton and carbon NMR spectra were taken of every fraction and compared with published chemical shift values of the known compounds from *Clathria* sp. Comparing chemical shift values helps to determine the presence of similar chemical structures and functional groups between the literature compounds and those isolated from sample 0609. Additionally, molecular weights from mass spectra were searched in MarinLit software and compound hits were compared with corresponding ^{1}H and ^{13}C NMR to confirm the presence or absence of

previously discovered marine natural products. Neither the comparison of NMR spectra or the MarinLit search yielded any compound matches.

DISCUSSION

Therapeutic Potential

Based on results of the *Artemia* sp. lethality test, fractions FD, FH, and DMH are the most promising extracts in terms of potential medicinal applications. LC_{50} values of these extracts against *Artemia* sp. ranged from 11 to 13 µg/mL. Assay results are not directly comparable when different model organisms are used but, to give these results a little perspective, Clathriol, previously isolated form a *Clathria* species was found to inhibit histamine release in peritoneal mast cells by 72 percent and inhibit the activation of human peripheral blood neutrophils by 76 percent at a concentration of 30 µM (14.4 µg/mL) [20]. Another compound previously isolated from a *Clathria* species, Microcionamide B, showed cytotoxicity toward two human breast tumor cell lines with LC_{50} values of 177 and 172 nM (0.155 and 0.151 µg/mL, respectively) [23]. Further biological testing of these extracts should be pursued on other organisms or cell lines to further explore their bioactivity.

Anti-fouling Potential

Results of the *Bugula neritina* bioassay suggest that fraction DMH is the most promising anti-fouling candidate. A subsequent assay with *Balanus amphitrite* barnacles further confirms that the crude DMH fraction and all sub-fractions have anti-fouling properties; all of these fractions showed 100 percent inhibition of settlement and metamorphosis while controls did not yield similar results. The ideal anti-fouling compound has both strong anti-settlement properties and low cytotoxicity. In this way, the compound works to prevent unwanted growth by other organisms without being toxic to the surrounding environment. For example, a boat coating containing anti-fouling components should prevent barnacle growth but not be toxic to other marine life. Fractions DMH 2, 3, and 4 all showed high cytotoxicity and should be re-tested at lower concentrations to determine if anti-fouling properties are retained at lower cytotoxicities. DMH 1,

on the other hand, appears to be a good anti-fouling candidate at the test concentration of 1.0 mg/mL.

Chemical Profiling

^{1}H, ^{13}C, and mass spectrometry data were reviewed and compared with literature reports of each of the compounds previously isolated from *Clathria*, however none of the known compounds were found in the examined fractions of sponge 0609. A lack of repeatable compound isolation might suggest that initial field taxonomy is incorrect and 0609 is not a *Clathria* sp. Furthermore, the fact that no matches were found through the MarinLit database suggests that the major compounds found in the 0609 extracts have not previously been identified or catalogued.

Complete structure elucidation was not feasible due to difficulties purifying extracts primarily because of very small sample size. The compounds closest to being pure FD/FH 4B H1-3 were not available in masses adequate to obtain a ^{1}H or ^{13}C NMR spectra. The largest of these fractions was 10 mg and was still insufficient for structure elucidation.

If further isolation were pursued it would be worthwhile to attempt to further separate fraction DMH 2 or DMH 4. Crude fraction DMH showed the best anti-settlement properties and sub-fractions 2 and 4 hold the compounds with the largest masses and most interesting functional groups as seen by NMR.

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