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# Alternative Extraction Method of Guanidine Metabolites from Marine Sponge, Ptilocaulis spiculifer

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# Alternative Extraction Method of Guanidine Metabolites from Marine Sponge, *Ptilocaulis spiculifer*

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**TABLE OF CONTENTS**

**ABSTRACT**: Marine sponges are known for their use of biologically active allelopathic compounds. With almost every species of sponge having been shown to produce some chemical with medicinal properties, their survival is becoming increasingly important. Current extraction methods used by research teams require a large sample relative to the size of the sponge, which threatens the survival of the organism.[1] *Ptilocaulis sp.*, or the orange tree sponge, is known to produce guanidine metabolites. This derivative has demonstrated biological activity against cell lines of leukemia, uterine, and cervical cancer.[2, 3] In this study we have developed a method for the chemical extraction of active metabolites from the ambient water containing a sponge. Preliminary data suggests the metabolite was found both using the traditional methods and using the water extraction. The organic extracts were used to test against L1210 leukemia cells for biological activity.

## INTRODUCTION

Marine sponges have been a great source of medically relevant compounds since their first use in the ancient world as anything from an analgesic to a cutaneous applicable cough suppressant. In 1949, Bergmann and Feeney started the derivation of compounds from these natural resources with their discovery of three nucleosides from a Caribbean sponge.[4] The sharp increase in using natural products for treatments or cures in more serious illnesses over the last 50 years has created a need to extract these chemicals with a greater yield and higher frequency.[5]

Current extraction techniques involve procurement of a marine sponge either by direct harvest from the sea or from a commercial vendor followed by a solid/liquid extraction. The Heys group slightly altered an extraction method with dichloromethane originally used by the Patil group on the sponge *Batzella sp.*[6] The group completed a $CH_2Cl_2$:MeOH (1:1) extraction on a freeze-dried sample of the sponge and further extracted by triturating the red solid with three solvents. The solvent fraction containing the main guanidine metabolites, which are the focus of this research, was the $CH_2Cl_2$ extract.[3]

**Figure 1**. The orange tree sponge, *Ptilocaulis spiculifer*, we used in this study.

The sponge of interest in this study, *Ptilocaulis spiculifer*, the orange tree sponge shown in Figure 1, is native to the Caribbean. Although it

is available for purchase in the United States, the scarcity of the sponge makes it difficult to find. *Crambe crambe* is another sponge that has shown promise in medicine for the secondary metabolites that this species produces, but it is found in the Mediterranean.[7] Although not as rare, all marine sponge species that produce medically relevant metabolites run the risk of being over harvested. Focusing on species like this will make the results of such an alternative extraction that much more important for the survival of the sponges. Metabolites produced by each species are similar, but ptilomycaulin A has been shown to be the major metabolite of *Ptilocaulis spiculifer*, which is the focus of this study. Metabolites of interest that *Crambe crambe* produce are the family of crambescins and crambescidins, which are also mentioned in this study and shown to be produced in smaller quantities by *Ptilocaulis spiculifer*.

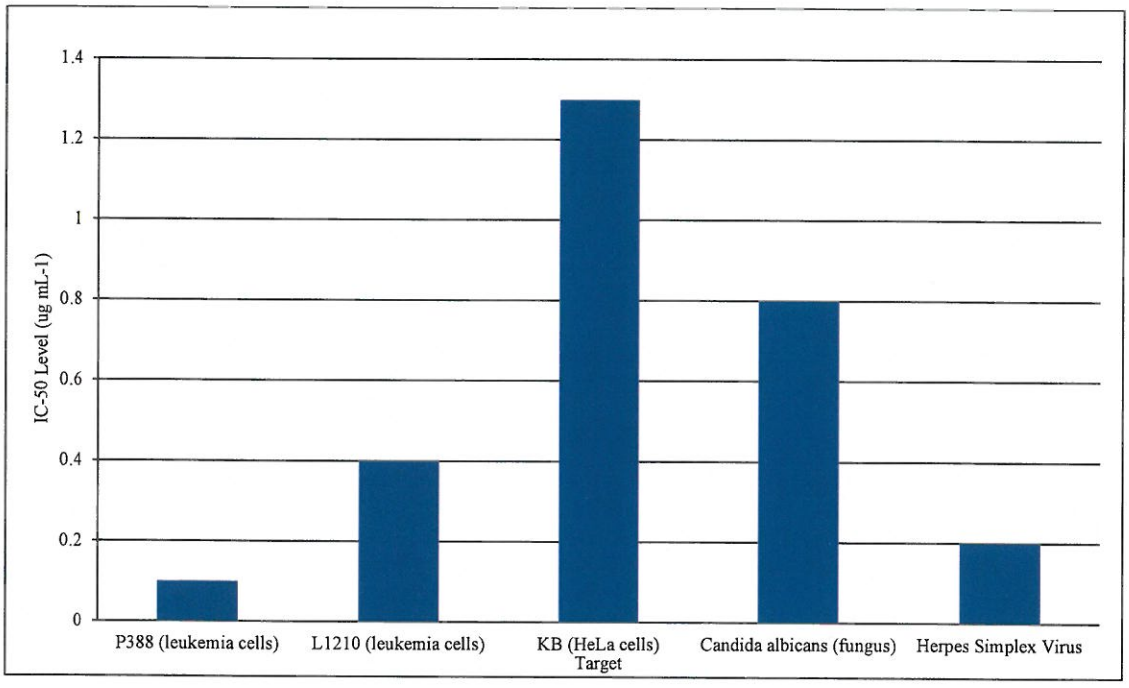
Unfortunately, current extraction methods, hereby referred to as *traditional extraction methods*, end in the loss of wildlife due to exposure to organic solvents and maceration of the sample. Utilizing this method for treatment of the human population creates a reliance on a non-renewable process that would not have the efficacy to reproduce enough viable sponges to replace those lost to extraction. In order to extract just one gram of the anticancer agent ET 743, it takes almost one metric ton (wet weight) of the marine sponge *E. turbinate*.[4] These extraction methods also select for a general group of molecules bearing similar physical properties (i.e. solubility), and therefore the extract is not a pure sample of the desired metabolite. Purification of these extracts often leads to sub milligram quantities of isolated metabolites using the traditional methods.

Due to the small quantities isolated by extraction, many of the miracle compounds are currently being synthetically designed. These processes require expensive reagents and produce large quantities of hazardous waste.[8] These synthetic methods do generate a more pure product, but there are fallbacks. Not only do these methods require a large amount of various reagents and waste disposal, but they also require extended amounts of time, frequently more than 6 months of work for one batch, and the yield is often less than 1% conversion from the initial reagents to the product.

*Ptilocaulis spiculifer* and *Crambe crambe* are known to produce secondary metabolites crambescidin-800 and crambescidin-816 (**1**, **2**), batzelladines A and B (**3**, **4**), and ptilomycaulin A (**5**), which are all examples of those allelopathic compounds that have been isolated using the traditional extraction method (Figure 2). In particular, ptilomycaulin A has a known biological activity against L1210 (lymphocytic leukemia) cells, P388 (leukemia), KB (HeLa contaminant, carcinoma and papilloma), as well as antifungal activity against *Candida albicans* and antiviral activity (HSV).[2, 3] *Ptilocaulis spiculifer* was chosen because the major metabolite, as its name implies, is ptilomycaulin A. We are most interested in this metabolite because it has a high activity against cell lines mentioned above at low concentrations. Laboratory limitations made it impossible to study the effects on fungal or viral targets. The KB cell line was not chosen because it contained multiple strains of cancerous cells. P388 and L1210 both served as viable options for the target in this research, but due to the difficult growth environments necessary for P388, it was decided that the target would be the L1210 cell line.

Levels of IC-50, or half maximal inhibitory concentration, for each of the inflictions are shown in Table 1. IC-50 generally refers to a way of measuring the activity of agents in inhibiting different pathogens.[9] In this case, it specifically measures the concentration of the compound at which fifty percent of the target cells are immobilized or destroyed.

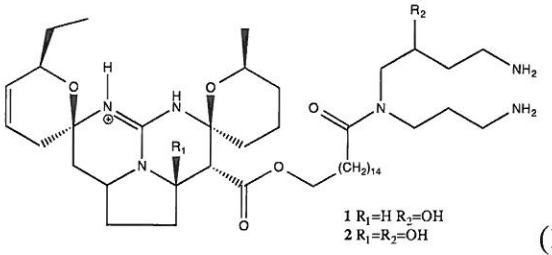
**Table 1**. Biological activity for ptilomycaulin A as reported by Heys et. al.



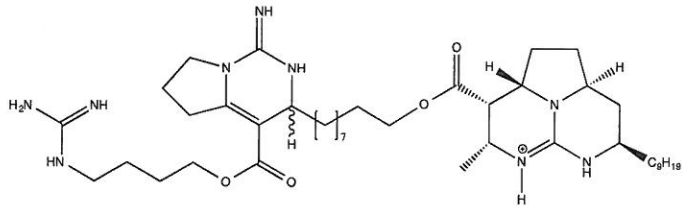

This project focuses on creating a more ecologically friendly way to extract these chemicals that do not end in the loss of the organism. This could possibly mean a lower financial input as well as a self-sufficient system with continual extraction for higher yields. Many of the compounds of interest are allelopathic compounds meaning they are secreted from the sponge to cease the growth of nearby organisms ensuring the space necessary for the expansion of the sponge itself.[4] Due to the purpose of these compounds, it is reasonable to assume that the metabolite would have a significant

concentration in the waters surrounding the sponge. This makes liquid/liquid extraction of the ambient waters a viable alternative to the traditional solid/ liquid extraction.

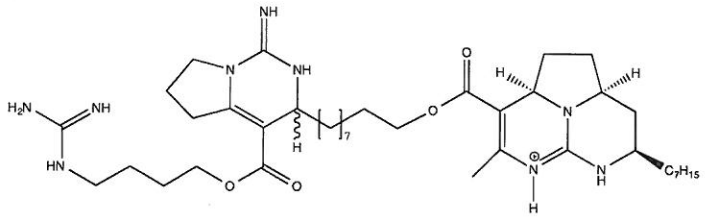
With the ambient water extraction, we propose it would be possible to have *Ptilocaulis spiculifer* growing in a salt-water tank and use the water from a routine water change to extract those compounds for application against L1210 leukemia cells. Some of the known metabolites listed above are shown below in Figure 2.



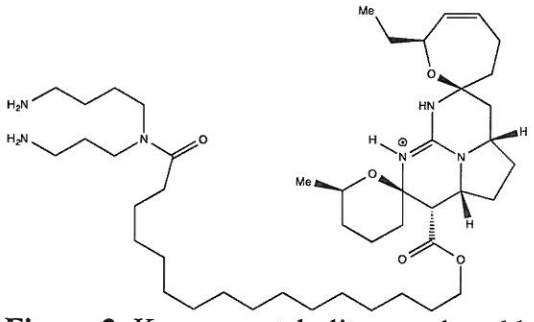

(**1**, **2** – crambescidin 800 and 816)



(**3** – batzelladine A)



(**4** – batzelladine B)



(**5** – ptilomycaulin A)

**Figure 2**. Known metabolites produced by *Ptilocaulis spiculifer*.

## EXPERIMENTAL SECTION

Extraction was completed using water from a tank that contained two sponges of the species *Ptilocaulis spiculifer*. After extraction, the fraction was concentrated and analyzed using liquid chromatography – mass spectrometry (LC-MS) and biological assays. As mentioned before, ptilomycaulin A was originally thought to be the main metabolite of this species. So, the ambient water extraction was derived based on the traditional extraction method used for ptilomycaulin A specifically. Each process is described in detail below.

When first working with the sponge, we also decided to complete the traditional solid/ liquid extraction process with one of the sponge specimens in order to ensure the presence of the chemical and also to verify the most efficient identification method of the metabolites. This process is described in the Traditional Extraction section.

***Traditional Extraction.*** A full red tree sponge (*Ptilocaulis spiculifer*; Aquacon) was placed into a liquidizer with 1:1 dichloromethane to methanol and the entire specimen was ground up until segments of the organism resembled small shavings. The sample was filtered and the liquid concentrated under vacuum and stored below -4°C until analysis.

***Ambient Water Extraction.*** Approximately 200 mL dichloromethane was added to the extractor to cover the lower transfer line (Figure 3). Marine tank water was then added to the reservoir of the continuous liquid-liquid extractor until the DCM was pushed over and filled the round bottom flask halfway. This point was reached with about 1 L of tank water.

The DCM was heated at reflux then condensed through the water sample accumulating the organic metabolites in the round bottom flask with each pass. Accumulation occurs in the round bottom because the compound does not evaporate with the solvent and therefore the solvent is simply reused to continually extract the compound from the sample on the left. Figure 4 shows a diagram of the movement of solvent and compound using this glassware. Solvent was allowed to circulate through the sample for at least 24-hours. The prolonged and continuous exposure with the extraction solvent was expected to give the highest possible yield

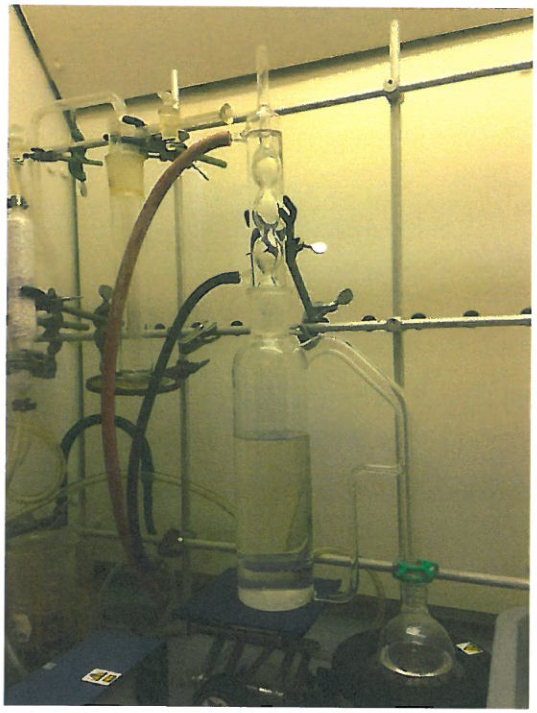


**Figure 3**. Glassware set up for the overnight reflux of tank water with dichloromethane for the liquid-liquid extraction of organics.

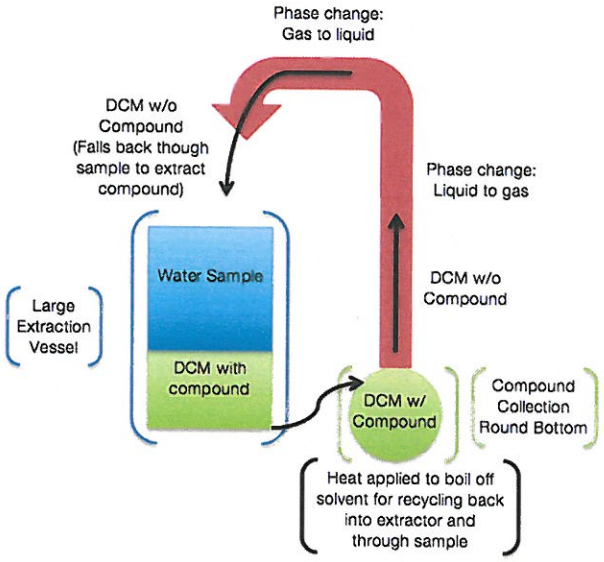



**Figure 4**. Diagram illustrating the extraction process of the compound from the water sample. Green brackets represent the round bottom in which the compound is accumulated; blue brackets represent the large partition of the extraction glassware where the sample and DCM interact. Red areas show where the DCM flows, but the compound does not; green areas shown where the compound is present in the DCM; the blue area represents the water sample.

from the water source. After extraction for 24 hours, the tank water sample was replaced with another liter to continue collecting the compound from a fresh sample. Therefore, each ambient water extraction sample was derived from 2 liters of the tank water.

When the extraction was complete, the round bottom flask was removed from the apparatus and its contents concentrated under vacuum. The concentrated oil was stored below -4°C until preparation for analysis with LC-MS or a cytotoxicity assay.

**Table 2.** This table shows all of the known mass spectral data about the metabolites of interest in this study. Included as well are physical properties of the compounds after extraction or synthesis in other labs.

| Compound | Exact Mass (amu) | Reported Fragmentation | Physical Properties |
|---|---|---|---|
| *Crambescidin-800* | 801.62 | None | Yellow oil[11] |
| *Crambescidin-816* | 817.61 | None | Proposed to be the same as above[10] |
| *Batzelladine A* | 768.58 | 655, 637, 609, 474, 350, 332, 304, 114 | Colorless gum[6] |
| *Batzelladine B* | 738.59 | 318, 302, 274 | Colorless, amorphous solid[6] |
| *Ptilomycaulin A* | 786.18 | None | No color[3] |

***LC-MS/UV/Vis.*** A liquid chromatograph with electrospray ionization-mass spectrometry detection was used to analyze the samples for the presence of one of the metabolites. Exact masses, fragmentation data, as well as physical properties can be found in Table 2. Samples were prepared by dissolving the residue in the round bottom flask with acetonitrile (MeCN). Each sample was eluted on a C18 reverse phase column

using a MeCN mobile phase. Samples were also detected using UV/Vis at wavelength 272 nm. Results are present in Figures 5 and 6 of the results section.

***Cytotoxicity Assay.*** A Pierce LDH Cytotoxicity Assay Kit (Thermo Scientific) was used with L1210 (lymphocytic leukemia) cells to analyze all samples. Samples were dried under high vacuum to ensure an accurate mass of the mixtures.

Samples were then dissolved in 75:25 PBS:dimethyl sulfoxide (DMSO) for final assay concentrations of 2X, 1X and 1/2X (X=0.4 $\mu g\ mL^{-1}$), where 1X is the IC-50 of ptilomycaulin A against L1210 leukemia cells, as cited by *Heys et. al.*[3] This concentration was used because the initial hypothesis was that the extracted metabolite would be ptilomycaulin A. DMSO of equal volume to the highest volume used in the extract dilutions, media, and 100% lysis were also measured for control purposes.

## RESULTS AND DISCUSSION

Overall, the ambient water extraction method was successful in extracting metabolites produced by the sponge from the surrounding water. Although the major metabolite expected, ptilomycaulin A, from this sponge was not found, there is evidence supporting the presence of crambescidin-816. Results from both LC-MS and the cytotoxicity assays supported the presence of the crambescidin. Complete characterization of the metabolites extracted was not possible within this lab. However, using current literature with the results gathered here it was possible to determine the major composition of the oil extract. Results showing this are discussed below.

***Extractions***

For the traditional extraction method the extract had a dark orange color. This was suspected to be mainly due to the orange color of the sponge. However, the ambient water extraction yielded a yellow oil. It should be stressed that no part of the sponge was collected in the extraction method, and that only water was used in the extraction process. Therefore the color of the extract must come from something that was extracted from the water.

***LC-MS/UV/Vis***

Using the traditional extraction method and the ambient water extraction, LC-MS and LC-UV/Vis measurements were taken to analyze the extracts. The full chromatograms for both the traditional extraction and the ambient water extraction are shown in Figures 4 and 5.

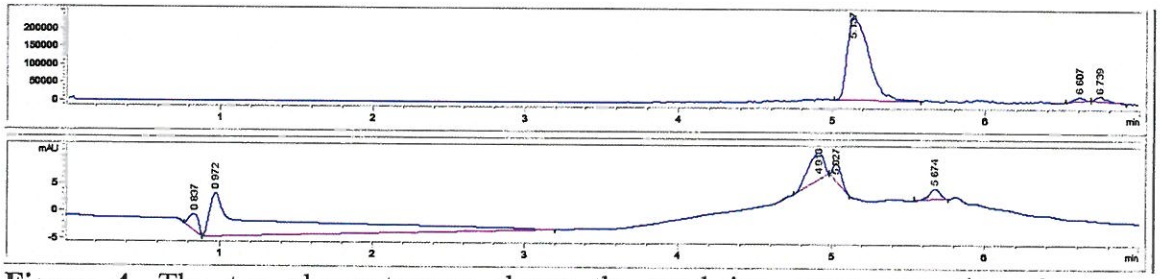


**Figure 4**. The top chromatogram shows the total ion count over time for the metabolites extracted by the traditional extraction method. The UV-Vis chromatogram (272 nm) during the same time course is below the mass spectrum.

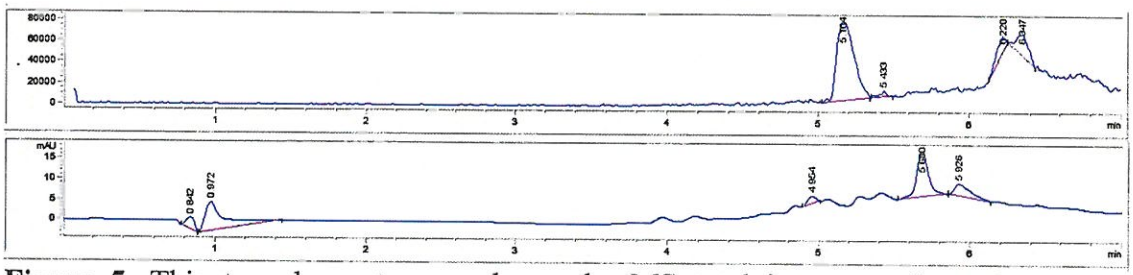


**Figure 5**. This top chromatogram shows the MS total ion count from the extract obtained using the ambient water extraction method. The UV-Vis chromatogram (272 nm) is included on the bottom chromatogram showing the scan during the same time course.

It is clear based on these spectra that there is a major metabolite extracted with both methods that elutes at 5.1 minutes.

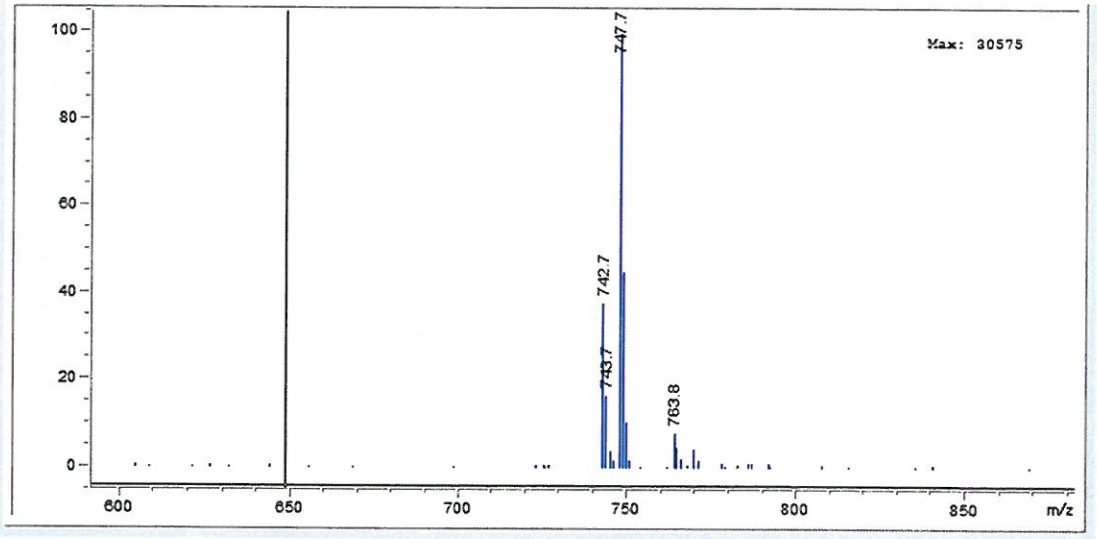



**Figure 6**. Mass spectrum from the ambient water extraction at 5.155 minutes (the elution time of the major metabolite from both the ambient water extraction and the traditional extraction method).

Figure 6 shows the mass spectrum obtained at 5.155 minutes from the ambient water extract, which had similar fragments to the traditional extraction sample; only one was shown here. The major ion peak at 747.7 did not seem to match any fragmentation suggested for ptilomycaulin A. However, there were possible fragmentations for crambescidin 800, crambescidin 816, and batzelladine A that would result in the peaks denoted above: the most likely of these are one of the crambescidin compounds. Batzelladines are not as likely here because there are published fragmentation patterns for both of the batzelladines and the other fragments, as listed in Table 1, are not present in this chromatogram. In addition, the color of the batzelladines do not match the yellow oil obtained from the ambient extraction method.

***Cytotoxicity Assay***

Cell cytotoxicity was determined by comparing the cell death found in the samples to the cell death when in the presence of lysis buffer, which was assumed to be 100% lysis.

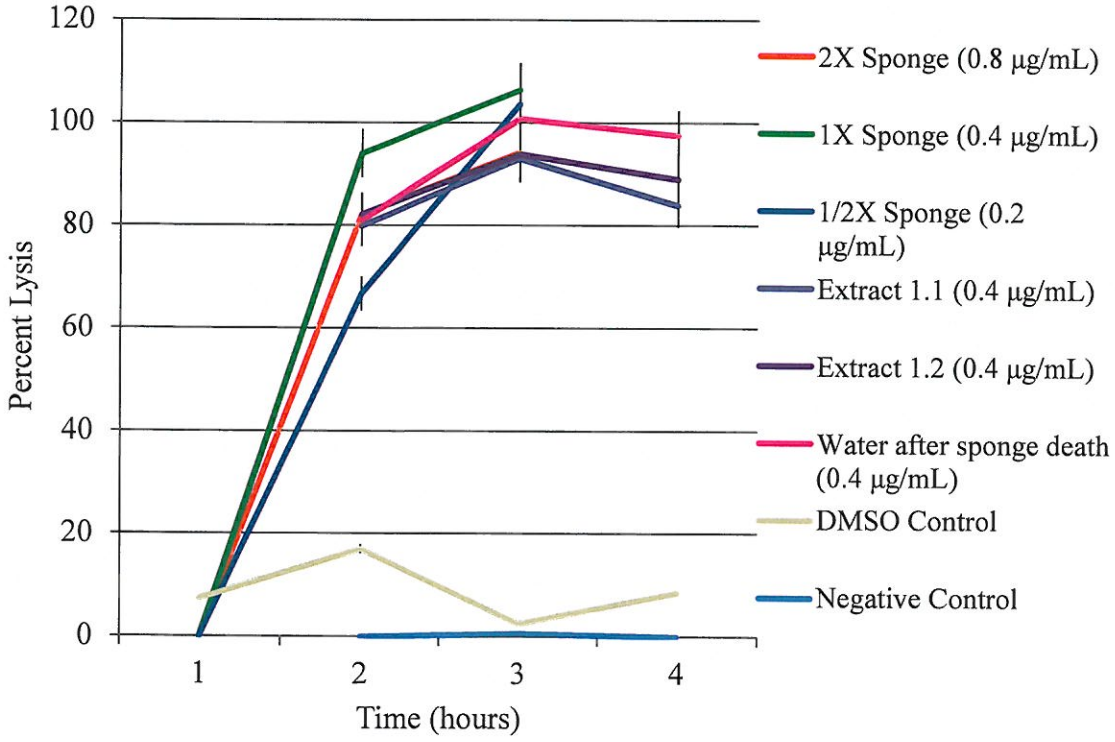



**Figure 6**. Cell cytotoxicity when comparing the activity of the extracts to that of 100% cell lysis when cells were exposed to lysis buffer. Sponge samples denote samples derived from the traditional extraction method. Extract 1.1 and 1.2 denote separate extracts taken from tank water changes. DMSO served as a control because the extracts were dissolved in DMSO, which is known to have a slight cytotoxicity effect. Error bars show 5% error.

Sponge samples denote sample that were obtained using the traditional extraction method. These were expected to show cell lysis due to the use of this method in literature, and thus they served as positive controls for the extraction samples. Concentrations noted as 2X, 1X and 1/2X (X=0.4 µg $mL^{-1}$), correspond to the CC

(cytotoxic concentration)-50 (CC-50 = 1X) of ptilomycaulin A against L1210 leukemia cells as cited by *Badinger et. al.* Again, as mentioned in the experimental section, the ptilomycaulin A CC-50 was used because the initial hypothesis was that ptilomycaulin A would be extracted from the ambient water. Extracts 1.1 and 1.2 are samples derived from the ambient water extraction method. The negative control was a salt-water sample taken from a fish tank. This was considered to be a good control because the tank in which the sponges were held were pre-treated with three fish and two hermit crabs to prepare the water for the sponges: as the data shows, the presence of the wildlife did not add cytotoxic activity to the extraction samples.

## CONCLUSIONS/ FUTURE WORK

The identity of the compound could not be completely characterized, however it is proposed that the most likely metabolite isolated was crambescidin 816. *Coffee et. al.* report that crambescidin 800 is a yellow oil when in the salt form, but the only difference between crambescidin 800 and crambescidin 816 is a hydroxyl group, so it is proposed that the color of crambescidin 816 will also be yellow; the same color as the oil extract obtained using the ambient water extraction method.[11] While the color of the extracts do not provide the compounds identity, the activity of the extracts were much higher than expected for ptilomycaulin A.

In addition to the color of the compound extracted, *Jares-Erijman et. al.* reported crambescidin 816 as the most abundant of all of the crambescidin metabolites.[12] In another year, *Jares-Erijman et. al.* reported that the hydroxyl group not present on ptilomycaulin A and crambescidin 800 that is present on crambescidin 816 infers greater cytotoxicity effects.[13] This is relevant because the cytotoxicity levels

noted in the assays were much higher at the 1/2X concentration than literature reports for ptilomycaulin A. Therefore, due to the color of the extract, the absence of fragments from the batzelladines and ptilomycaulin A, and the cytotoxicity activity of the compound, it is proposed that crambescidin 816 is the metabolite extracted using the ambient water extraction method. Further testing to fully determine the presence of this metabolite would be purification by preparation chromatography coupled with single- and multi-dimensional, single- and multi-nuclear NMR analysis. NMR data for all metabolites already exist in literature, and it would therefore be a viable determination of the metabolite.

Further application of this method could be used for a large-scale operation to optimize extraction. For example, a large tank could have a continual flow of water to an extraction chamber where the compound is extracted and further processed while the water that is extracted could be returned to the tank. The organic extraction would act as a carbon filter that is commonly present in many saltwater tanks. Over the amount if time it takes for synthesis to be completed, it would be interesting to compare the amount of compound extracted from the water to that of the yield of the synthesis.

Actual application of the compound to cancer in a murine model system would be the next step after purification. Peptoid drug delivery systems have been shown very effective in the delivery of guanidinium compounds to the nucleus.[14] Having an efficient extraction system with a constant extraction of product like this will make the isolation of these biologically relevant, allelopathic compounds much easier, more affordable, and with little to no hazardous by-product. Metabolites like the ones listed are not the only allelopathic compounds produced by marine organisms. Application of

this water extraction system could potentially reach any marine organism with an allelopathic protection mechanism accomplishing environmentally friendly extraction of natural marine compounds. Changing the solvent used for extraction could also change the metabolite isolated creating numerous options for optimizing this method for different metabolites, or groups of metabolites, to be extracted. Although the original target of ptilomycaulin A was not believed to be extracted, this method was successful in extracting the allelopathic metabolites of *Ptilocaulis spiculifer* from the ambient water.

**ACKNOWLEDGEMENTS**

I would like to acknowledge all those who made this research project possible during my senior year. I would like to thank Dr. Andrew Yeagley and Dr. Amorette Barber for their continued guidance and hands on involvement with the project over the last year: their dedication and intellect has not gone unnoticed. I would also like to thank Longwood University Departments of Chemistry and Physics, and Biology and Environmental Science for funding as well as providing a research space. Additionally, I would like to thank the Cormier Honors College for their financial and emotional support, especially Dr. Alix Fink. I would like to thank Dr. Melissa Rhoten for intellectual conversation for the furthering of the project as well as undying support of me as a researcher and a student. Finally, I would like to thank my parents, Chester and Deborh Barnett, and my fiancée Kellen Dunn, for their continued support throughout life and my college career.

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