Partial Isolation and Chemical Characterization of Cytotoxic Compounds in Freshwater Sponges

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ABSTRACT

Marine sponges are known to produce or sequester compounds useful for defense against predation and to prevent fouling by parasites. Many of the compounds extracted from marine sponges have been shown to possess useful antitumoral properties. The present study investigates two species of freshwater sponges, *Eunapius fragilis* and *Ephydatia muelleri*, for possible cytotoxic compounds as determined by brine shrimp microassay. Results show that both species have cytotoxic compounds extractable in methanol and at least two cytotoxic compounds may be present in *Eunapius fragilis*, both of which are extractable in hexane. The present study provides strong support for continued natural product research on freshwater sponges.
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INTRODUCTION

The sessile characteristic of sponges has led to the evolutionary development of defensive compounds to protect against predation and fouling by invasive parasites, especially in competitive waters such as coral reefs (Proksch, 1994). Many of these compounds produced by sponges have proved to be of benefit to humans (Wright, 1998). For example, spongistatin 1, isolated from marine sponges, has been shown to inhibit mitosis in cancerous, kangaroo rat cells (Bai et al., 1993). Axinastatin 1, also isolated from a marine sponge, was shown to have cytostatic effects on human cancer cells (Pettit et al., 1994). Niphatevirin, a glycoprotein isolated from the marine sponge Niphates erecta, has shown strong activity in anti-HIV bioassays (O’Keefe et al., 1997). Knowledge of the structures of these compounds opens possibilities for the synthesis of related compounds with enhanced activities.

Previous natural products research has focused on marine sponge species; however, little research has been conducted using freshwater species of sponges. In previous studies by Daehnke (2006) and Bania (2007), cytotoxic compounds extracted from freshwater sponges were detected with modified brine shrimp assays as described by Meyer et al. (1982). A cytotoxic compound from Spongilla lacustris and Ephydatia muelleri was reported to be soluble in methanol and have a molecular weight between 3,000 and 30,000 Daltons (Bania, 2007). The present study attempts to confirm the presence of the methanol-soluble, cytotoxic compound from Ephydatia muelleri and Eunapius fragilis while further characterizing and isolating the cytotoxic compound.
An efficient and inexpensive brine shrimp assay was shown by Ferrigni and McLaughlin (1984) to be an effective way to isolate fractions of natural extracts that have a high probability of testing positive for activity in more expensive anti-tumoral assays. Thus, the brine shrimp assay is useful for preliminary screening. The present study used a microplate brine shrimp assay adapted from Solis et al. (1992) to detect the presence of cytotoxic chemicals. The microplate technique allowed for more reproducibility and less statistical error than previous freshwater sponge studies.

While results did not support the presence of a methanol-soluble, cytotoxic compound, at least two hexane-soluble, cytotoxic compounds were detected. Silica gel chromatography was used to partially isolate these compounds. Some preliminary HPLC analysis showed inconclusive results.
MATERIALS AND METHODS

Collection, identification, and storage

Sponge samples were scraped from rocks found in Salmon Lake, Missoula County, Montana (47° 4.03'N, 113° 23.33'W (NAD27)). Samples were transported to the laboratory in lake water in a cooler and identified as *Eunapius fragilis* and *Ephydatia muelleri* by spicule inspection. The sponge samples were gently blotted with paper towels to remove excess water, placed in plastic containers and stored at -20° C. For some experiments, sponge samples collected in July 2006 and stored at -20° C were used.

Extraction

The general outline of the extraction is a modified procedure taken from Wright (1998) and includes homogenization of the sponge sample in solvent using a mortar and pestle and sonication, centrifugation of homogenate, and collection and evaporation of supernatant. Methanol and hexane (HPLC grade, Fisher) and ethanol (200 proof, AAPER) were used as extraction solvents on both *Ephydatia muelleri* and *Eunapius fragilis*.

Twelve to thirteen grams of frozen, hydrated sponge sample were chopped into small cubes and placed into a 4° C mortar with 35 mL of cooled solvent. The sample was crushed and the resulting homogenate was transferred to centrifuge tubes. The tubes of homogenate were placed into a Branson Model 1510 sonication bath filled with 2° C water and sonicated for 10 min. The sonicated homogenate was centrifuged for 5 min at 5000 X g and 4° C. Supernatant was collected and the pelleted tissue residue was
resuspended in another 35 mL of hexane. The sonication and centrifugation were repeated four times, each time removing and combining the supernatant fractions. For hexane extracts, the combined supernatant was washed with water three times in a separatory funnel and dehydrated with sodium sulfate. Because methanol extracts tended to be turbid, they were ultracentrifuged at ~300,000 X g for 30 min at 4° C in order to pellet particulates. Pellets from the ultracentrifugation were resuspended in 50 mM HEPES. Both pellet resuspension and supernatant were assayed.

Silica gel chromatography

A silica-hexane slurry was poured into a 1.5 cm diameter column to a bed height of 8.5 cm. One milliliter of hexane extract was loaded followed by 50 mL of each of the following eluents: hexane, hexane-ethyl acetate 3:1, hexane-ethyl acetate 1:1, hexane-ethyl acetate 1:3, ethyl acetate, and ethyl acetate-methanol 1:1. Each 50 mL eluent flowed at a rate of 90-110 drops per min and was collected in a separate round bottom flask. Elution fractions were put on a vacuum rotoevaporator to remove solvents. The residues were then reconstituted in 0.5 mL of hexane, with the exception of the ethyl acetate-methanol fraction, which was reconstituted in 0.5 mL of methanol. Each was assayed for activity against brine shrimp.

Solvent residue controls for the shrimp assay were created by evaporating 50 mL of the eluent solvents in a round bottom flask on a rotoevaporator. Any solvent residues were reconstituted in hexane (methanol for the ethyl acetate-methanol 1:1 eluent) and assayed for activity against brine shrimp.
Brine shrimp assay

Brine shrimp eggs were hatched in a solution of 1.5% sodium chloride buffered with 1 mM HEPES at pH 7.5. Eggs were incubated in a warm water bath at 25°C for 48 hours under heavy aeration. Stage II and III instars were collected using a bright light to attract active, phototropic nauplii.

Microplate assays were set up by adding 25 μL, 12.5 μL, and 6.25 μL of sample into different wells on a 96 well, polystyrene plate. Each sample/concentration combination was repeated in three separate wells for triplicate testing. Additionally, respective control solvents were added to three wells. The sample and control solvents were allowed to evaporate to dryness overnight at 4°C. One hundred microliters of 1.5% sodium chloride/50 mM HEPES assay solution were added to each well along with 10-20 active nauplii. The microplate was allowed to incubate at 25°C for 24 hours.

Dead shrimp were counted with a dissection microscope. Shrimp that showed no appendage twitching for more than five seconds were recorded as dead. After the number of dead shrimp in each well was recorded, 200 μL of methanol was added to each well in order to kill the remaining shrimp. After 15 min, a total shrimp count for each well was recorded. Mortality rates were reported as the percentages of dead shrimp to total shrimp.

HPLC

Samples were run on C-18 and C-8 columns with a solvent flow of 1.25 mL/min on the Buck Scientific BLC-10. Sample peaks detected by UV absorption were collected for assay. Elution solvents included methanol and acetonitrile-water (7:3).
Statistical Analysis

To calculate mortality rate, the number of dead shrimp was divided by the total number of shrimp for each well. For each sample/concentration set (triplicate), the mortality rates were averaged. To show significance of activity, sample set mortality rates were graphed with standard error bars and compared to control set mortality rates.
RESULTS

Confirmation of Activity in Methanol Extract

This experiment was conducted to confirm the cytotoxic activity of methanol extracts from samples of *Ephydatia muelleri* and *Eunapius fragilis* that were collected the previous year and kept frozen. While the methanol extracts from both species showed activity (Fig. 1), the samples appeared to be cloudy. The cloudy nature suggested that the extracts were methanol suspensions rather than methanol solutions.

![Brine shrimp mortality due to methanol extracts prepared from two species of freshwater sponges, *Ephydatia muelleri* and *Eunapius fragilis*.](image)

Ultracentrifugation of Methanol Extracts

Ultracentrifugation was performed on the cloudy methanol extract in order to sediment the fine particulates in suspension. Ultracentrifugation supernatants as well as non-ultracentrifuged extracts of both *E. muelleri* and *E. fragilis* were assayed for this experiment. Results showed a decrease in activity against brine shrimp in the extract supernatants when compared to non-ultracentrifuged extracts (Fig. 2). Methanol extracts
from both *E. fragilis* and *E. muelleri* were cloudy as before; however the supernatants of the ultracentrifuged samples were transparent.

![Figure 2](image_url)

*Figure 2.* Brine shrimp mortality due to methanol extracts before and after ultracentrifugation.

**Analysis of Ultracentrifuged Pellet**

Due to the loss of activity in the supernatant from the ultracentrifugation experiment, analysis of the pellet was needed to determine if cytotoxic compounds were settling during ultracentrifugation. This experiment used methanol extract from *E. fragilis* only. The crude methanol extract was compared to both the ultracentrifuged supernatant and pellet (resuspended in 50 mM HEPES) (Fig. 3). Results show that the majority of the activity found in the crude methanol extract was recovered in the ultracentrifuge pellet and not the supernatant. The pellet resuspension was very cloudy.
Figure 3. Effects of ultracentrifugation fractions from *E. fragilis* methanol extract on brine shrimp mortality.

*Analysis of Various Extraction Solvents*

Because the activity found in crude methanol extract did not truly go into solution, I attempted to find a solvent that could fully dissolve the active compounds in *E. fragilis*. Hexane, ethanol, and a solution of methanol-water (3:1) were tested as extraction solvents. While all three extracts showed cytotoxic activity (Fig. 4a), only the hexane showed activity as a transparent solution.

Figure 4a. Effects of various solvent extracts from *E. fragilis* on brine shrimp mortality.
Ethanol and methanol-water extracts were cloudy and required ultracentrifugation. The activity for the supernatant and pellet resuspension for these extracts are summarized in Figure 4b and compared to the hexane extract solution. The hexane extract required no ultracentrifugation, rather, it was washed and dried as described.

![Graph](image)

**Figure 4b.** Effects of ultracentrifuged fractions from various *E. fragilis* extracts on brine shrimp mortality.

**Analysis of Fractions from Silica Gel Chromatography**

Silica gel chromatography was performed in order to further isolate the cytotoxic compounds and give some insight into the polarity of the molecules. No residues from the hexane-only (1st) fraction were visible after the solvent was fully evaporated. The hexane-ethyl acetate (3:1) (2nd) fraction contained the greatest amount of visible residues after evaporation of the solvent (crystalline white and off-white solids) but showed no apparent activity against brine shrimp (Fig. 5). The hexane-ethyl acetate (1:1) (3rd fraction) left a small amount of oily, yellow, viscous droplets as residue. This was one of the most active fractions (Fig. 5). The hexane-ethyl acetate (1:3) and ethyl acetate
fractions (4th and 5th) had almost no visible residues and no apparent activity. The ethyl acetate-methanol (1:1) (6th) fraction had a little off-white solid residue lining the round bottom flask. This fraction also showed high levels of activity (Fig. 5).

![Figure 5](image)

**Figure 5.** Effects of silica gel chromatography fractions on brine shrimp mortality. *Crude hexane extract* refers to the sample loaded onto the silica column. All other fractions were collected off the column.

**HPLC Analysis of Fraction 3 (hexane-ethyl acetate 1:1)**

HPLC of the hexane-ethyl acetate (1:1) fraction provided a more sensitive technique to potentially further separate compounds of similar polarity. The hexane-ethyl acetate (1:1) fraction residues of the previous silica gel chromatography experiment were run through a Higgins Analytical C-8 column with methanol as the eluent. The sample material came through the column as a single, broad peak with no resolution. The eluent of this broad peak was collected and assayed. The sample showed a 93% mortality rate against the brine shrimp.
Figure 6 shows a UV absorption graph of the hexane extract residues* before silica chromatography compared to the hexane-ethyl acetate (1:1) fraction residues. Both samples were run on Higgins Analytical C-18 columns with acetonitrile-water (7:3) as eluent. Neither of these samples was assayed for activity.

![Graph showing UV absorption of hexane extract residues before silica chromatography compared to hexane-ethyl acetate fraction residues.](image)

**Figure 6.** HPLC absorption spectrum of crude hexane extract (blue line) compared to hexane-ethyl acetate (1:1) fraction from silica chromatography (red line). Eluent was acetonitrile-water (7:3).

* The hexane extracts used for this HPLC analysis sat at 4°C for over six weeks. During that time, the extract changed color from clear yellow to cloudy, pale white. It is possible that the extracted compounds oxidized or otherwise changed chemical structure.
DISCUSSION

Previous studies by Daehnke (2006) and Bania (2007) showed that three species of freshwater sponges, *S. lacustris*, *E. fragilis* and *E. muelleri*, contain molecules that show activity in a brine shrimp mortality assay. The present study confirms the presence of at least one biologically active compound in *E. muelleri* and at least two compounds in *E. fragilis*. While the chemical nature of the molecules remains ambiguous, it is clear that three species of freshwater sponges contain compounds capable of killing brine shrimp within 24 hours of exposure.

The two aforementioned studies used assay methods with larger volumes of both assay brine solution and sample extract. The microplate assay method (Solis et al., 1992) used in this study confirmed similar results with higher number of replications in less time. I suggest this assay procedure for future work.

Methanol was an effective solvent to extract one or many of the biologically active compounds. However, upon ultracentrifugation, the supernatant showed a significant decrease in activity while the pellet retained activity. This suggests that the active compounds in the methanol are possibly large, dense macromolecules or physically associated with large molecular complexes capable of being pelleted under 300,000 X g or are not completely soluble in methanol. While this study did not pursue the isolation of cytotoxic compounds extracted in methanol, it may prove to be an interesting direction for future study.

Previous attempts to extract cytotoxic compounds from *S. lacustris* and *E. muelleri* using diethyl ether yielded no activity (Bania, 2007). In the present study, I was
able to extract significant activity from *E. fragilis* using both hexane and ethanol. However, upon ultracentrifugation, the ethanol extract seemed to lose activity in both the supernatant and the pellet. Since the hexane extracts were not ultracentrifuged, it cannot be assumed that the hexane extract would not have lost activity as well. The cytotoxic compounds in the hexane extract must have been substantially non-polar given that the compounds persisted in the hexane after consecutive washes with water. If possible, future work with hexane-soluble compounds would best be extracted using lyophilized sponge samples to avoid the mixing problems encountered with hydrated samples.

Upon running hexane extracts through a silica column, two fractions with distinct activity were recovered (Fig 5). The fact that the two active fractions were separated by two inactive fractions indicates that there are at least two different compounds in a hexane extract from *E. fragilis* that cause lethality in brine shrimp assays. The procedure by which these active fractions were obtained proved to be consistently repeatable. Since the active compounds came off the column in solvents that were more polar than hexane (ethyl acetate solutions), it is plausible that the compounds were also present in the methanol suspension of previous experiments. The silica chromatography process also removed a good portion of non-active compounds from the hexane extract. The hexane-ethyl acetate 3:1 fraction (2nd) had by far the most residues left in the collection flask, yet none of the residues in this fraction showed activity against brine shrimp. This indicates that some purification took place by using the silica column.

Preliminary work with HPLC indicated that the hexane:ethyl acetate 1:1 (3rd) fraction residues could be collected from the C-8 column using methanol and still be active in a brine shrimp assay. While no peak resolution was achieved in this study,
future HPLC work could prove to be very effective in further isolating the cytotoxic compounds.

Since activity in the brine shrimp assay has been suggested to indicate activity in potato antitumor assays (Ferrigni & McLaughlin, 1984), the active fraction from this study should be tested on the potato disc assay. Activity in this type of assay would be an encouraging next step for potential antitumor drug discovery. Additionally, samples of isolated active compounds need be sent through proper mass spectral instrumentation in order to identify compounds or functional groups.


Daehnke, D.E. (2006) Screening for antibiotic, antifungal, and antitumoral compounds in natural products extracted from freshwater sponges [BA thesis], Carroll College, Helena, MT.


