INVESTIGATION OF MICROBIAL EXTRACTS

AS A SOURCE OF NEUROLOGICAL AGENTS

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ABSTRACT

This study tested natural products from cyanobacteria from the Red Sea in Egypt and marine tunicates from the Algoa Bay in South Africa. Products from marine organisms have been used as lead compounds for the synthesis of new drugs because of their biological activity mechanism. Specifically, these products may contain compounds that display voltage-gated sodium channel activity, a feature of some medications for nervous system conditions such as epilepsy (Neurontin, Tregetol, Keppra) or cardiac arrhythmias (Norpace, Xylocaine, Rythmol). Initially, products and fractionations were screened in mouse neuroblastoma cells (Neuro-2A) and cytotoxic activity was measured in an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay after a 24 hour period. The most active extracts identified in the cytotoxicity activity assay were selected as candidates for voltage-gated sodium channel activity measurements. This assay was a modified form of the initial MTT assay that exposed cells to known neurotoxins, ouabain and veratridine, to create a high concentration of sodium ions inside the cells. Extracts with voltage-gated sodium channel activity would either rescue the cells by inhibiting the channel, or activate the channel and kill the cells. The cytotoxicity assay identified 21 products, crude and fractionated, as somewhat cytotoxic.

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1. INTRODUCTION

A neurological disorder is any general irregular condition involving the brain and/or spinal cord (National Library of Medicine, 2009). Generally, these disorders are developed from diseases such as epilepsy, Parkinson's and Alzheimer's, or a serious injury such as a car accident (National Library of Medicine, 2009). Depending on the cause, symptoms of neurological disorders include a loss of coordination, decreased ability to communicate verbally, memory loss, depression and pain (National Library of Medicine, 2009). The specific cause of these symptoms and development of the disorder comes from a loss of neurons and glial cells (Lindvall & Kokaia, 2006). Most of these symptoms can be moderated and treated, but there are very few actual cures for those diagnosed with neurological disorders (Lindvall & Kokaia, 2006). Someone suffering with a neurological disorder may not notice the problem until it is too late to make lifestyle changes, causing concern for health organizations because neuronal damage is irreversible (National Library of Medicine, 2009). There is hope that stem cell research can help this by stimulating the formation of lost neurons and glial cells (Lindvall & Kokaia, 2006).

Neurological disorders are becoming increasingly prevalent throughout the world, yet are still considered low-priority diseases by public health organizations compared to other diseases. The increased prevalence of neurological disorders may be linked to changes in our environment such as stressful lifestyles (Fureman BE, 2002). Research scientists are aware that variables such as genetic makeup, age, diet and many other variables represent risk factors for neurological disorders, but specific mechanisms causing the disorders are

not well understood (Fureman BE, 2002). There is a pressing need for an increase in research to understand the specific mechanisms that cause a neurological disorder in order for neurologists to diagnose disorders earlier and more accurately. Advancements in genetic research and drug discovery projects targeting neurological disorders will help decrease the prevalence of neurological disorders by identifying people at high-risk, lower the chances of its development, and possibly lead to a cure for those suffering with a neurological disorder.

Drug discovery projects, while lengthy and high risk, are necessary because of the potential benefits of developing a drug. A drug discovery project is usually initiated by testing numerous compounds in a basic screening assay and narrowing down the number of interesting compounds to a select few for extended studies (Feher & Schmidt, 2003). Once a target compound has shown some significant value in these tests, it may be considered for further evaluation and clinical trials (Feher & Schmidt, 2003). Despite technological advancements and improved understanding of molecular and physiological processes, the drug discovery process can take many years and generally displays a low "success rate" compared to other research projects (Feher & Schmidt, 2003). However, even in an unsuccessful drug discovery project, eliminating compounds that did not show success in screenings provides information that may help other related projects.

The lucrative benefits of a unique and effective drug have led scientists to pursue a variety of sources for drug discovery purposes. Natural product compounds derived from the secondary metabolism of living organisms are significant starting points for most

drug discovery projects because of the diversity of these compounds and the success they have shown as starting points for drug development (Feher & Schmidt, 2003). Many different organisms can contribute natural products for drug discovery projects, but marine invertebrates, microorganisms and plants are considered important providers of natural products (Newman & Cragg, 2006). If a natural product shows potent and/or selective activity in a drug discovery project, its chemical structure is analyzed and used as a blueprint to create derivatives of the structure that may be developed with combinatorial chemistry techniques (Feher & Schmidt, 2003). To highlight the significance of natural products, in 2007, a study reported on the origin of 974 new drugs from 1981-2006 (Newman & Cragg, 2006). This report found that 63% of the drugs in the report were either directly derived from, or semi-synthetically derived from, a natural product (Newman & Cragg, 2006).

Bacteria and fungi have provided numerous different lead compounds for drug discovery. These microorganisms provide plentiful and diverse natural products used in competition with other microorganisms and macro organisms (Simmons, Andrianasolo, McPhail, & Gerwick, 2005). These natural products had to evolve over time to give the microorganisms a distinct survival advantage over an opposing organism (Levy, 1994). In medicinal research, natural anti-microbial products have primarily been used as a template to derive pharmaceuticals. Penicillin is an antibiotic drug class composed of a group of different antibiotic compounds originally derived from the *Penicillium* fungi (Wainwright & Swan, 1986). As the first antibiotic pharmaceutical, its discovery led to a

significant decrease in the prevalence of many different diseases caused by bacteria, including syphilis and *Staphylococcus* infections (Wainwright & Swan, 1986).

The applications of anti-microbial products have expanded beyond bacterial infections. Aplidin is derived from *Tridemnum solidum* and is used an anti-cancer agent (Medina, et al., 2008). Aplidin inhibits ornithine decarboxylase, an enzyme critical in the process of tumor growth and angiogenesis (Medina, et al., 2008). Asperlicin is a compound derived from *Aspergillus alliaceus* and is an antagonist of cholecystokinin (Chang, et al., 1985), which is a peptide hormone that controls appetite (Chang, et al., 1985).

As a starting point for a drug discovery project, Dr. Kerry McPhail of the Oregon State University Department of Pharmaceutical Sciences collaborated with other scientists stationed in Egypt and South Africa to investigate natural products that may display neurological activity. She targeted the Red Sea specifically to collect cyanobacteria because these bacteria have previously provided an array of natural product leads for drug discovery projects, and the Red Sea has a unique environment, providing numerous cyanobacteria (Banaja, 1990) that have not been investigated. Cyanobacteria have been important microorganisms for drug discovery research because they have provided lead compounds for some antitumor pharmaceuticals (Simmons, Andrianasolo, McPhail, & Gerwick, 2005). The Red Sea has a rich and diverse ecosystem due to its high salinity and large coral reef along its shallow portions (Lieske, 2004). The organisms that live in the Red Sea were forced to adapt to the high salt concentrations that most organisms do

not find suitable to survive (Lieske, 2004) . The cyanobacteria that live in the Red Sea have unique characteristics, making the location enticing for drug discovery efforts.

Algoa Bay, which lies along the East Cape coast off Port Elizabeth in South Africa, was a second target destination for Dr. McPhail. Marine tunicates were collected from this area to study their natural products. Tunicate natural products have not been studied as much as products from other filter-feeding marine organisms such as sponges and corals, primarily due to the difficulty of storing them and analyzing their taxonomy (Simmons, Andrianasolo, McPhail, & Gerwick, 2005). Tunicates have still produced lead compounds that led to approved anticancer agents, such as Ecteinascidia-743 and Aplidin, and need further investigation (Simmons, Andrianasolo, McPhail, & Gerwick, 2005). Aplidin has shown promise to help treat those suffering from multiple myeloma, a cancer of plasma cells within bone marrow (Mitsiades, et al., 2008). Algoa bay has an unusually high number of tunicates on its rocky reefs compared to other coral reefs, making it an ideal location to collect tunicates (Mitsiades, et al., 2008).

Dr. Jane Ishmael's laboratory received crude extracts from cyanobacteria and tunicates to investigate their cytotoxicity activity and their effects on voltage-gated sodium channels. A cell line that originated from neuroblastomas was used as models to measure both types of activity. A neuroblastoma is a neuroendocrine tumor that originates within the developing sympathetic nervous system (Thiele, 1998). Cells from neuroblastomas have a very high density of voltage-gated sodium channels, similar to neurons (Thiele, 1998).

The cell line chosen as a model was the Neuro-2A cell line, which comes from neuroblastomas developed in mice (Thiele, 1998).

Voltage-gated sodium channels are involved in the generation of action potentials, a cellular signaling mechanism neurons use to control the nervous system (Moyes & Schulte, 2005). To create an action potential, an initial stimulus will cause a change in membrane voltage potential within the cell (Moyes & Schulte, 2005). The membrane potential change will open the voltage-gated sodium channels, causing a rush of positively charged sodium ions to enter and depolarize the inside of the cell (Moyes & Schulte, 2005). The positively charged inside of the cell opens neighboring voltage-gated potassium channels and causes positively charged potassium ions to exit the cell and restore the voltage balance (Moyes & Schulte, 2005). To restore the balance of sodium and potassium ions, Sodium-Potassium ATPase uses ATP to actively shuttle sodium ions outside of the cell and potassium ions into the cell (Moyes & Schulte, 2005). This cycling of ions propagates along the neuron and is primarily used to send a signal from neuron to neuron (Moyes & Schulte, 2005).

This drug discovery investigation of the tunicate crude extracts and fractions will be used to guide the isolation of pure compounds responsible for the observed biological activity on cells structurally similar to neurons, and specifically having the mechanism of activating or inhibiting the voltage-gated sodium channels that neurons contain. Current medications for nervous system conditions such as epilepsy (Neurontin, Tregetol, Keppra) or cardiac arrhythmias (Norpace, Xylocaine, Rythmol) work by inhibiting or

activating voltage-gated sodium channels located in the neural plasma membrane (Feher & Schmidt, 2003). These medications help moderate neural synapses and allow the nervous system to function normally. The screening of the natural products collected from the Red Sea and South Africa will provide information on which products to pursue as possible lead compounds for future pharmaceuticals.

2. MATERIALS AND METHODS

2.1 Collection of Marine Organisms

Dr. McPhail collected cyanobacteria in the Red Sea for five days in shallow reef habitats, deep oceanic environments and mangroves. For each collection, three samples were prepared when possible for chemical investigation, taxonomic analysis via genetic procedures, and cultivation. Eleven different taxa were collected for chemical extraction. The cells were drained of all water and preserved in a small amount of 70% isopropanol. They were then stored at -30ºC until needed. Dr. Diaa Youseff, Suez Canal University, was involved with producing crude extracts from the cells, following an established protocol. For South Africa, 176 different tunicates were collected by Dr. Shirley Parker Nance, University of Port Elizabeth (Simmons, Andrianasolo, McPhail, & Gerwick, 2005). To date, 60 of the 176 tunicates have been extracted and the extracts were stored at -30ºC.

2.2 Cell Culture

Neuro-2A cells were cultured in RPMI-1640 media, supplemented with 1 mM sodium pyruvate, 50 μg/ml streptomycin, 50 μg/ml penicillin, 0.2% sodium bicarbonate and 10% fetal bovine serum (Manger, et al., 2003). All cell culture materials were purchased from VWR (Table 1.1). Cells were kept in an incubator with 5% CO_2 and 100% relative humidity. Cells were split at a cell subcultivation ratio between 1:3 and 1:6, depending on cell growth rates and future experiment plans (Manger, et al., 2003). Once the Neuro-2A cells reached around 75% confluency, they were considered ready for harvesting and 0.25% trypsin was used to resuspend the cells off the culture plate.

2.3 Cytotoxicity Screen

Neuro-2A cells used for the cytotoxicity experiments were cultured in 24 well plates at approximately 450,000 cells per well in a volume of 500 μl. After transferring the cells to a 24 well plate, the plate was incubated for a 24 hour period to ensure that the cells adhered to the well. Following the twenty-four hour incubation, each well was treated with an extract sample at 30 μg/ml. All extracts were stored in a freezer at 3 μg/μl in 100% DMSO. To prepare the working samples for experiments, 20 μl of the stored extract was added to 80 μl of media to make a 0.6 μg/μl sample in a test tube. Each well was replenished with 475 μl of fresh media and 25 μl of the working sample was then added to each well; making a four well test for the sample. The final concentration of sample in each well was 30 μg/ml and DMSO levels were 1%. Samples were left in the well for a 24 hour treatment period.

A colorimetric MTT, 3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide, assay was incorporated to determine cell viability (Alley, et al., 1988). Yellow MTT is a reagent that is reduced to a purple salt when added to cells with active mitochondria (Mossman, 1983). The insoluble purple salts dissolve by adding acid isopropanol, creating a colored solution (Mossman, 1983). The color of the solution is dependent on the amount of purple salts; it can be quantified by measuring its absorbance with a spectrophotometer. Comparison with an untreated control determines the percentage of living cells in the treated well.

After the 24 hour treatment period, the medium was removed and each well received 300 μl of fresh media. For this assay, MTT was dissolved in PBS at 5 mg/ml. 30 μL of MTT dissolved in PBS was added to each well creating a 1:10 ratio of MTT reagent to media (Alley, et al., 1988). The cells were then left in an incubator for 1 hour to allow the yellow MTT to be reduced to purple salts. After the incubation period, the medium with MTT was removed and 300 μl of 0.04 M HCl in isopropanol was added to each well to dissolve the purple salts (Alley, et al., 1988). The plate was put on a shaker for 10-15 minutes to allow the salts to dissolve. Once the salts dissolved, the contents of each well were transferred to a 96 well plate to be read on a spectrophotometer. The plate was read at a wavelength of 570 nm and absorbance was reported (Alley, et al., 1988).

2.4 Screening for Voltage-Gated Sodium Channel Activity

Neuro-2A cells were treated with a specific concentration of two neurotoxins, ouabain and veratridine. Veratridine is a voltage gated sodium channel activator and its presence will cause a spike in the sodium ion concentration inside the cell (Yannick, Romey, & Lazdunski, 1980). Ouabain is a sodium-potassium pump inhibitor and its presence will prevent the sodium-potassium pump from removing sodium ions within the cell (Yannick, Romey, & Lazdunski, 1980). The combination of these two neurotoxins causes an extremely high sodium ion concentration in the Neuro-2A cells. Extracts that show voltage gated sodium channel activity may either activate the sodium channel and further increase cell proliferation, or inhibit the channel and rescue the cell from sodium ion caused proliferation.

2.4.1 Screen for Voltage Gated Sodium Channel Activation

After a 24 hour seeding period identical to the cytotoxicity assay, Neuro-2A cells were exposed to 30 µM ouabain and 300 µM veratridine (Alley, et al., 1988). Wells used for testing were then exposed to a specific extract sample at 30 µg/ml and incubated for 24 hours. Brevetoxin, a neurotoxin known to activate voltage gated sodium channels, was used as a positive control for these experiments (Kogure, Tamplin, Simidu, & Colwell, 1988). Comparisons between wells with brevetoxin, wells with only ouabain and veratridine without extract, and wells with no toxins or extracts were used as controls for comparisons. The MTT assay was used to determine the absorbance and calculate cell viability for the assay.

2.4.2 Screen for Voltage Gated Sodium Channel Inhibition Activity

After a 24 hour seeding period identical to the cytotoxicity assay, Neuro-2A cells were exposed to 50 µM ouabain and 500 µM veratridine (Alley, et al., 1988). Wells used for testing were then exposed to a specific extract at 30 µg/ml and incubated for 24 hours. Tetrodotoxin, a neurotoxin known to inhibit voltage-gated sodium channels, was used as a positive control for these experiments (Kogure, Tamplin, Simidu, & Colwell, 1988). Comparisons between wells with tetrodotoxin, wells with only ouabain and veratridine without extract, and wells with no toxins or extracts were used as controls for comparisons. The MTT assay was used to determine the absorbance and calculate cell viability for the assay.

2.5 Optimization of General Cytotoxicity Screening

The cytotoxicity protocol was optimized to preserve supplies. A multi-channel pipette allowed the use of conducting the protocol on 96 well plates. All cell-culturing supplies were identical to what was used in the initial 24 well cytotoxicity screening except a multi-channel pipette was substituted for a single-channel pipette. Each well contained approximately 45,000 Neuro-2A cells per well and 50 μL of media when seeded. After the 24 hour incubation period, each experimental well had the old media removed, and was treated with a specific sample at 30 μg/μL in fresh media. Generally, 8 wells were used for each sample on a plate, depending on supply and the experimental goals. For an 8-well treatment, 4.5 μL of the extract stored in 100% DMSO was diluted in 22 μL of media to make 26.5 μL of a working sample. This working sample was added to 423.5 μL of media in a multi-channel pipette reservoir, putting the extract at 30 μg/μL and 1.0% DMSO.

3. RESULTS

3.1 Red Sea

3.1.1 Crude Extracts

From the Red Sea cyanobacteria, nine of the crude extracts were tested for cytotoxicity testing. Ehu-05/28/07-3, a crude extract from cyanobacteria collected from the hull of a wrecked ship, was the most active crude Red Sea extract. Ehu-05/28/07-3 decreased cell viability to 21% (Table 3.1) in comparison to the untreated control. The remaining crude samples produced little decrease in cell viability, with 81%-113% of the cells remaining viable, displaying slight to no activity (Table 3.1).

Ehu-05/27/07-3 (Ehu-5), an extract from a *Lyngbya* cyanobacterium (Table 2.1), was considered a priority extract by Dr. McPhail's lab and chosen for fractionation. The parent extract decreased the viability of Neuro-2A to 87%, suggesting it had very slight activity. While insignificant as a crude mixture, some of its subfractions showed much higher activity (Table 2.1, 4.1), suggesting that some components of this crude sample are biologically active.

3.1.2 VLC Fractions, Ehu-5(A-I)

The Ehu-05/27/07-3 (Ehu-5) crude extract was fractionated by polarity with normal phase vacuum liquid chromatography following a standard fractionation protocol (Figure 2.2). Of these fractions A-I, Ehu-5G and Ehu-5H were the most active. Ehu-5G decreased cell viability to 32%, and Ehu-5H decreased cell viability to 27% when compared to the untreated controls (Table 4.1). These results strongly correlated with

separate brine shrimp toxicity assay (McPhail, pers comm). Both Ehu-5G and Ehu-5H killed over 80% of the shrimp in the assay while all other tested samples showed less than 20% toxicity. Ehu-5H was selected for further fractionation based on the brine shrimp toxicity studies (McPhail, pers comm.). Of the other samples, Ehu-5E displayed moderate activity, decreasing cell viability to 58% (Table 4.1). All of the other fractionated samples decreased or increased cell viability to 84-112% (Table 4.1).

3.1.3 SPE Fractions Ehu-5H-(1-5)

The Ehu-5H VLC fraction was partitioned into five subfractions (Figure 2.2). Ehu-5H was fractionated by reverse phase solid phase extraction (SPE) and subfractions were labeled 1-5 based on the polarity of the solvent, 1 being most polar and 5 being least polar (Figure 2.2). Three of the five subfractions of Ehu-5H were very active in the cytotoxicity assays. Ehu-5H-1, Ehu-5H-2 and Ehu-5H-3 all decreased cell viability to below 45% (Table 5.1). Ehu-5H-3 in particular was very toxic, decreasing cell viability to 7% (Table 5.1) compared to the untreated control.

These results confirmed that the biological activity could be traced from the crude extract down to its fractionated forms. The bioassay-guided fractionation of crude extract Ehu-5 to subfraction Ehu-5H-3 identified the most active fraction of Ehu-5, and further fractionation with reverse phase high performance liquid chromatography (HPLC) of this semi-pure mixture has since resulted in the isolation of two active pure compounds (data not shown). This fractionation of the Ehu-5 crude extract to the Ehu-5H-3 SPE fraction identified the most active fraction of Ehu-5 (Figure 5.2).

3.1.4 VLC Fractions Ehu-4H-4 (A-G)

Sample Ehu-4H-4 was considered a priority sample for activity based on previous research (McPhail, pers comm). Crude sample Ehu-4 was fractionated by normal phase vacuum liquid chromatography and Ehu-4H was fractionated by reverse phase solid phase extraction (Figure 2.2). This active parent fraction was then subjected to HPLC to isolate the active pure compound.

The SPE fraction, Ehu-4H-4, decreased the Neuro-2A cell viability to 79% compared to untreated controls (Table 6.1). This activity, considered very slight, did not correlate with prior studies (McPhail, pers comm). Of the Ehu-4H-4 subfractions, HPLC fractions Ehu-4H-4B displayed significant activity against the Neuro-2A cells. Ehu-4H-4B decreased cell viability to 24%, making this a priority sample (Table 6.1). All of the other fractionations of Ehu-4H-4 decreased or increased cell viability between 83% and 115% (Table 6.1).

SPE fraction Ehu-4G-4 was tested and decreased cell viability to 74% when compared to untreated controls (Table 6.1). While not considered a priority, Ehu-4G-4 did show more activity on Neuro-2A cells than Ehu-4H-4 (Table 6.1).

3.1.5 VLC Fraction Ehu-1(A-I)

The final set of supplied Red Sea extracts was Ehu-1(A-I) (Figure 2.2). There was not an Ehu-1 parent crude extract provided for testing, only the VLC subfractions A-I (Figure

2.2). None of these first tier fractions were considered priority samples based on prior research (McPhail, pers comm). Only one of the extracts, Ehu-1H, displayed slight activity, decreasing cell viability to 72% when compared to untreated controls (Table 7.1).

3.2 South Africa

Of 32 crude extracts from tunicates collected form rocky reefs in Algoa Bay, South Africa, 9 of them as priority samples for testing, on the basis of their taxonomy (McPhail, pers comm).

3.2.1 Priority Samples

Of the priority samples, SAF04-55 was the only crude sample with strong activity against Neuro-2A cells. SAF04-55 decreased cell viability to 2% compared to the untreated controls (Table 8.1). Samples SAF04-19 and SAF04-62 showed moderate activity, decreasing cell viability percentages to 62% and 60% respectively (Table 8.1). Intriguingly, SAF04-65 increased cell viability by a very large amount (to 141%) when compared to the untreated controls (Table 8.1). This extract significantly increased cell growth compared to all the other SAF04 crude extracts.

3.2.2 Non-Priority Samples

None of the other 23 non-priority extracts displayed any significant activity. The most active of the non-priority extracts was SAF04-71 with 75% cell viability of Neuro-2A cells compared to the untreated control (Table 9.1).

3.3 Voltage-Gated Sodium Channel Control Experiments

Before testing the extracts for voltage-gated sodium channel activity in Neuro-2A cells, all of the controls were validated in separate experiments. Both voltage-gated inhibition and activation activity controls were tested with specific protocols in 3 separate 24-well plates of quadruplicates using Neuro-2A cells. All of the controls for both activation and inhibition experiments displayed the expected trend. The presence of ouabain and veratridine was expected to decrease cell viability and it did: 66% cell viability average for the activation experiment (Table 10.1) and 35% cell viability average for the inhibition experiment (Table 10.2).

The significant decrease in cell viability in the inhibition experiment was caused by the higher molarity of ouabain and veratridine in this experiment. As a positive control, brevetoxin, when present with ouabain and veratridine, successfully activated voltage-gated sodium channels and decreased the average cell viability to 55%, an 11% decrease compared to ouabain and veratridine alone (Table 10.1). The other positive control, tetrodotoxin, inhibited the voltage-gated sodium channels when present with ouabain and veratridine and increased the cell viability to 43%, displaying a 9% overall increase in cell viability compared to the ouabain and veratridine control (Table 10.2).

While all of the controls did display the desired trend, the protocol for the voltage-gated sodium channel activity suggested that the average increase or decrease of cell viability should about 30% higher or lower than the wells with only ouabain and veratridine.

These controls could not be established in the given timeframe and therefore unknown samples were not tested for voltage-gated sodium channel activity.

3.4 96 Well Cytotoxicity Results

3.4.1 VLC Fractions SAF04-23(A-I)

For the 96 well cytotoxicity screening using Neuro-2A cells, South Africa sample SAF04-23 was separated into 6 first tier fractions by normal phase VLC (Figure 2.2). Crude extract SAF04-23 and its subfractions did not display any significant activity in these experiments; the lowest cell viability was from the parent extract at 81% (Table 12.1). None of these samples produced significant activity levels in any of the assays and the are not recommended for future research with voltage gated sodium channels.

3.4.2 VLC Fractions SAF04-30(A-I)

SAF04-30 was fractionated into 7 smaller samples using normal phase vacuum liquid chromatography with solvents A-I representing the polarity of the solvent (Figure 2.2). The only sample that reported any significant cytotoxic activity against Neuro-2A cells was SAF04-30I, which generated a cell viability of 54.03% (Table 13.1). This sample showed strong activity. As for the rest of the fractionations, none of them produced any significant activity levels in any of the assays; cell viability was between 85.93% and 106.95% (Table 13.1).

3.4.3 VLC Fractions SAF04-60 (A-G)

SAF04-60 was fractionated into 7 fractions using normal phase VLC (Figure 2.2). The parent extract, SAF04-60, produced slight activity but all the subfractions produced very slight to no activity when tested for cytotoxicity to Neuro-2A cells (Table 14.1). SAF04-60 was a priority sample, but it showed no activity in the 24 well and the 96 well cytotoxicy screenings (Table 14.1).

3.4.4 Crude Extract SAF04-55

SAF04-55 decreased cell viability by over 95% (Table 8.1), although significant activity was not found in other assays (McPhail, pers comm). Thus, SAF04-55 was selected for fractionation and further analysis.

3.4.5 VLC Fractions SAF04-55(B-H)

SAF04-55 was fractionated into 7 VLC fractions (B-H) (Figure 2.2). The cytotoxicity assays in Neuro-2A cells continued to report strong activity from SAF04-55, but only slight to very slight activity from the resulting subfractions. In parallel assays of the crude extract and its subfractions, SAF04-55 displayed a 51% cell viability average (Table 15.1), much higher than the 2% cell viability observed in the early screenings of SAF04-55 before fractionation (Table 8.1). The VLC fraction with the most significant activity was SAF04-55G, generating a 76% cell viability (Table 15.1).

3.4.6 SPE Fractions SAF04-55G-(1-4)

SAF04-55G was fractionated into 4 samples by reverse phase SPE according to the standard protocol (Figure 2.2). Fractions SAF04-55G2 and SAF04-55G3 both showed

strong cytotoxicity activity to Neuro-2A cells. These fractions produced 58% and 66% cell viability, respectively (Table 16.1), making them both significant for future research. While these fractions were active, the decrease in cell viability was still much less than what the parent, SAF04-55, produced in the earlier screenings. The other SPE fractions of SAF04-55G were insignificant (Table 16.1).

4. DISCUSSION

The cyanobacteria from the Red Sea and the tunicates from Algoa Bay in South Africa both provided products that show promise as possible voltage-gated sodium channel activators or inhibitors. Seven different Red Sea products and four South Africa products displayed strong activity in MTT cytotoxicity assays that should be considered in all future projects. Twenty-two other crude extracts or fractions from both locations displayed slight to moderate activity in the MTT cytotoxicity assays and may also be considered for future projects.

Previous experiments similar to the MTT cytotoxicity assay used a classification scheme for cytotoxic activity and were incorporated for our testing (Goeger, pers comm). For cell viability, any extract that decreased viability by 16-25% was defined as very slight, 26-35% as slight, 36-45% as moderate and anything greater than 45% as strong. Using these classifications, the extracts with strong activity in the 24 well cytotoxicity assay results include Ehu-05/27/07, Ehu-5G, Ehu-5H, Ehu-5H1, Ehu-5H-2, Ehu-5H-3, Ehu-4H-4B, and SAF04-55. All of these extracts displayed enough activity to be considered for future testing (Table 17.1).

In the 24 well cytotoxicity assay, Ehu-5E, Ehu-5H-4, SAF04-19 and SAF04-62 all displayed moderate activity. Ehu-4G-4, Ehu-1H and SAF04-71 all displayed slight activity and Ehu-5C, Ehu-4H-4, Ehu-4H-4C, Ehu-4H-4E, Ehu-1E, SAF04-18, SAF04-59 and SAF04-70 all displayed very slight activity (Table 17.1). This group of extracts are not considered a priority based on this classification scheme; however if there is enough

time or resources, they should be tested in order based on the level of activity they displayed.

The optimization of the cytotoxicity assay to the 96 well format increased the number of samples that could be tested at a time. This allowed us to test an extract and its subfractions on the same plate. SAF04-30I, SAF04-55B, and SAF04-55G1 were identified as showing strong activity (Table 14.1, 16.1); SAF04-55G2 was identified as showing moderate activity (Table 16.1); SAF04-60, SAF04-30E, SAF04-30F, and SAF04-55G were identified as showing slight activity (Table 12.1, 14.1, 15.1); and SAF04-23 and SAF04-30G were identified as showing very slight activity (Table 13.1, 14.1). The strong activity extracts should be considered for future testing and followed up while the rest of the identified extracts may be considered for testing if there are enough supplies and time.

Over the course of this project, several issues were identified that may have affected the results. The percentage of DMSO in each well was higher than recommended. DMSO is highly toxic and recommended to be at levels below 0.5% to ensure that it is not a primary source of cytotoxicity. In both screenings, the measured DMSO level was 1.0%. Since the controls used in the assay did not contain any DMSO, we can not assume that DMSO did not have an effect on the results. For comparative purposes, however, each extract was tested at the same DMSO levels. While the actual cytotoxicity results may be skewed due to the DMSO, relative toxicity comparisons between the extracts can still be made.

Another possible source of error may have originated from the protocol used for the 96 well cytotoxicity assay. In the 24 well assay, the cells were incubated for a 24 hour period in their wells and then received fresh media immediately before being treated with extracts. For the 96 well assay, however, the extracts were added to fresh media prior to being added to the plate. The 96 well plate then had all media removed and each row was treated with media and extract at the same time. This meant that there was a time period of 10-15 minutes in which the Neuro-2A cells had no media. This amount of time may have also had an effect on the cytotoxicity results and may affect any comparisons made between the 24 well cytotoxicity assay and the 96 well cytotoxicity assay. However, comparisons made between the extracts for the 96 well cytotoxicity assay will still be accurate.

These cytotoxicity results may contribute to a future discovery of a natural product as a starting structure point for the development of a future pharmaceutical. Many of these extracts show potential as lead compounds, but more testing is needed in identifying the active components of the crude extracts. Future projects involving these extracts must include a more refined cytotoxicity assays with DMSO below 0.5% and sodium channel activity assays. If any extracts show high voltage-gated sodium channel activity, they should be targeted for more extensive assays. Examples of other studies may include looking at changed doses, time of exposure intervals, different cell lines, etc. in order to completely understand the mechanism of toxicity.

Previously, Coibamide A was identified as a lead compound for a possible future pharmaceutical for cancer treatment (Medina, et al., 2008). Coibamide A is a potent dipeptide that originated from a marine cyanobacterium collected near Coba Island, Panama (Medina, et al., 2008). This compound has shown cytotoxicity to human lung, central nervous system, and colon tumor cells. (Medina, et al., 2008). Currently, the synthesis of Coibamide A is still a work in progress because the cyanobacterium that produces Coibamide A is unable to survive in the laboratory setting (Medina, et al., 2008). Coibamide A was isolated via a similar bioassay-guided process as the extracts from the Red Sea and South Africa and is a model of where this project may lead.

ACKNOWLEDGEMENTS

I would like to express gratitude for my advisors who helped guide me through this project. Dr. Jane Ishmael from the Oregon State University Department of Pharmceutical Sciences was my primary mentor and she helped me through every step of this project. College of Pharmacy graduate student Andrew Hau was involved in teaching me laboratory techniques associated with the project and setting up experiments. Wanda Crannell from the College of Agricultural Sciences was my academic advisor and she helped me connect with Jane Ishmael for this project. Dr. Kerry McPhail from the Oregon State University Department of Pharmaceutical Sciences was the secondary advisor for this project and her work with obtaining the microbial extracts and fractionating them was integral for this project to be accomplished. The Undergraduate Research, Innovation, Scholarship & Creativity was crucial in funding supplies needed for the assay.

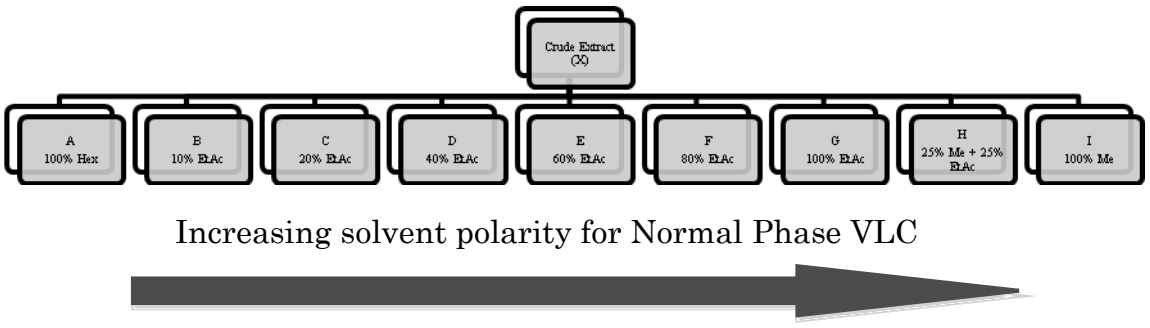
6. TABLES AND FIGURES

Table 1.1: Supplies Ordered for Experiments		
Supply	**Use for Experiment**	**Catalog # (VWR)**
RPMI 1640	Media	45000-12
Penicillin and Streptomycin	Antibiotics for media	45000-652
FBS	Serum for media	16777-014
Sodium Pyruvate	Supplement for media	45000-710
Sodium Bicarbonate	Supplement for media	45000-664
Trypsin 0.25%	Cell resuspension	45000-664
T-75 Flasks	Cell storage	15708-134
MTT Assay Kit	Kit to run tests	30-1010 (ATCC)
Tissue Culture Dishes	Cell storage	25382-166
Ouabain	Neurotoxin	100505-704
Veratridine	Neurotoxin	80055-496
Brevetoxin	Neurotoxin	80055-134
Tetrodoxin	Neurotoxin	80055-420

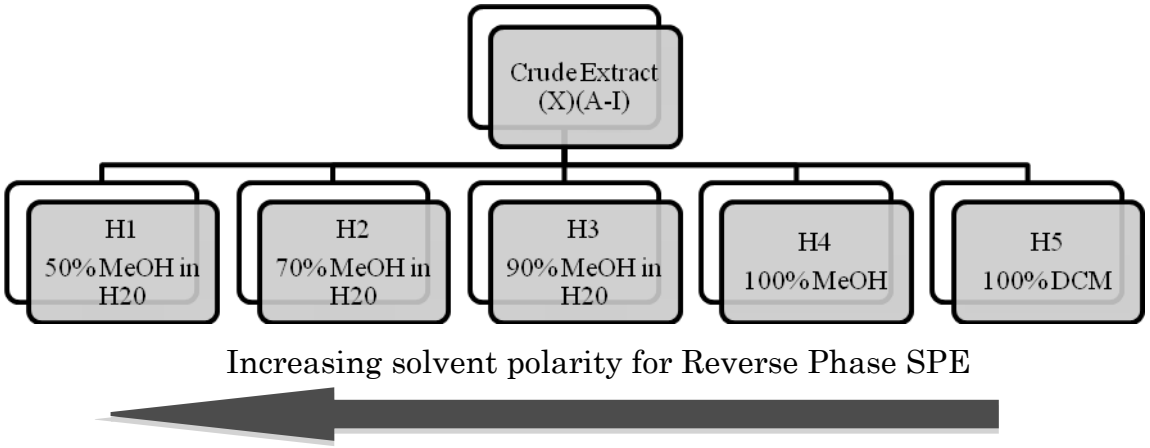
Table 2.1: Ehu-5 Crude Sample Description		
Sample Code	**Field Description**	**Amount Collected**
EHu-05/27/07-3	*Lyngbya*	1 X 500 mL
EHu-05/28/07-1	*Spirocoleus*	1 x 2L (start)
EHu-05/28/07-2	Cyanobacteria, bright orange	1 x 250 mL (start)
EHu-05/28/07-6	*Homothamnion*, green	1 x 500 mL (start)
EHu-05/28/07-3	Cyanobacteria, red	1 x 1 L (start)
EHu-05/29/07-3	Diatom-Cyanobacteria, red and gel like	1 x 1 L
ENq-05/31/07-1	*Scytonema*, green/brown and shaggy	1 x 2L (start)
ENq-05/31/07-2	*Schizothrix*, yellow and puffy	1 x 2L (start)
ENq-05/31/07-3	Cyanobacteria, dark brown	1 x 500 mL (start)

The Sample code refers to the date they were collected.

VLC Fractions



SPE Fractions



H=Hexanes, EtAc=Ethyl Acetate, MeOH=Methanol, DCM=Dichloromethane

Figure 2.2: Fractionation Schematic. Flow chart displays the solvents used in the fractionation process for the extracts. The code name for each extract follows the solvents used during the process. Fractions were labeled A-I to indicate the polarity of the solvent for VLC and 1-5 to indicate polarity for SPE.

Table 3.1: MTT Assay of Red Sea Extracts Ehu-5(A-I) Exposed to Neuro-2A cells for 24 hours						
Sample Code	**Absorbance Average**	**Standard Deviation**	**Trials**	**Standard Error**	**Control Average**	**Cell Viability**
EHu-05/27/07-3	0.227	0.036	4	0.009	0.260	87.30%
EHu-05/28/07-1	0.251	0.012	4	0.003	0.260	96.73%
EHu-05/28/07-2	0.282	0.029	4	0.00725	0.260	108.57%
EHu-05/28/07-6	0.212	0.026	4	0.0065	0.260	81.42%
EHu-05/28/07-3	0.057	0.002	4	0.0005	0.266	21.33%
EHu-05/29/07-3	0.241	0.038	4	0.0095	0.266	90.41%
ENq-05/31/07-1	0.238	0.012	4	0.003	0.266	89.57%
ENq-05/31/07-2	0.297	0.026	4	0.0065	0.266	111.56%
ENq-05/31/07-3	0.302	0.033	4	0.00825	0.266	113.35%

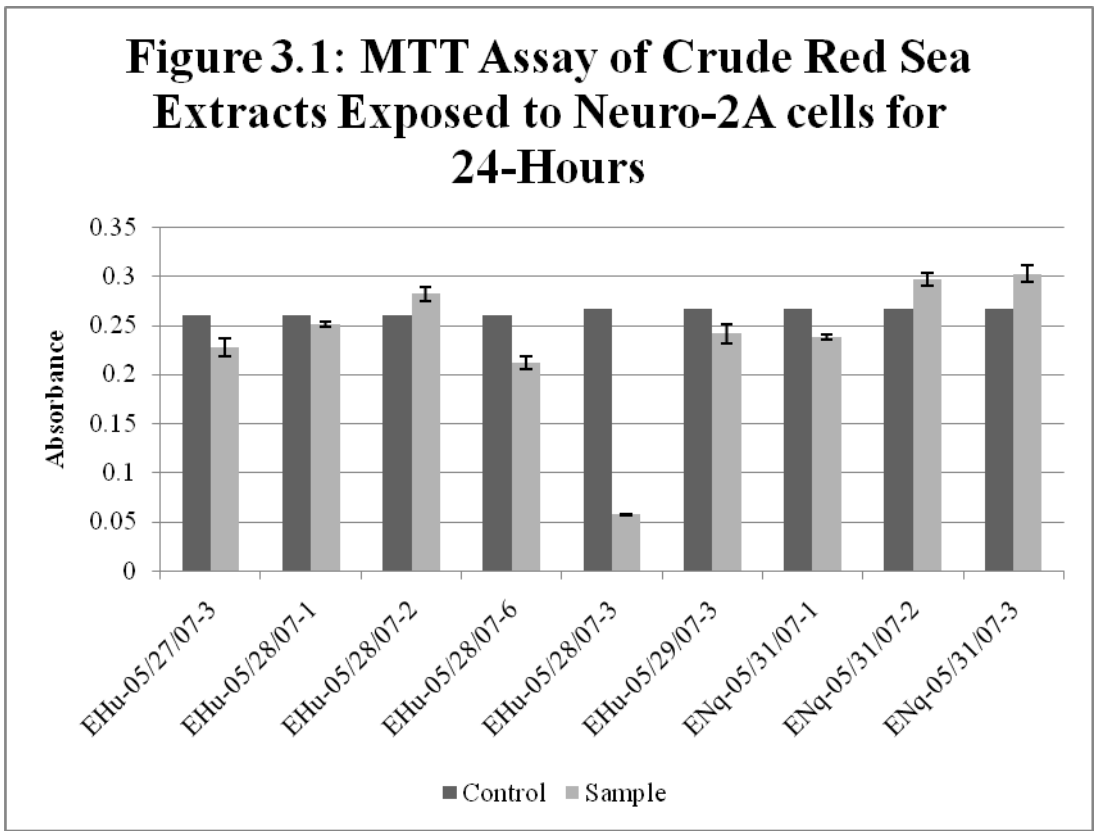


Figure 3.1. Histogram displays absorbance readings for Neuro-2A cells treated with crude Ehu-5 samples for 24 hours with an MTT assay, (Table 1.1). Controls for each sample were completely untreated and cultured on the same plate. Error bars represent the standard error of the mean.1

Table 4.1: MTT Assay of Red Sea Extracts Ehu-5(A-I) Exposed to Neuro-2A cells for 24 hours						
Sample Code	**Absorbance Average**	**Standard Deviation**	**Trials**	**Standard Error**	**Control Average**	**Cell Viability**
Ehu-5A	0.220	0.026	4	0.0065	0.222	98.99%
Ehu-5C	0.186	0.036	4	0.009	0.222	83.99%
Ehu-5E	0.129	0.047	4	0.01175	0.222	58.29%
Ehu-5G	0.070	0.020	4	0.005	0.222	31.68%
Ehu-5H	0.059	0.025	4	0.00625	0.222	26.61%
Ehu-5I	0.279	0.036	4	0.009	0.250	111.51%

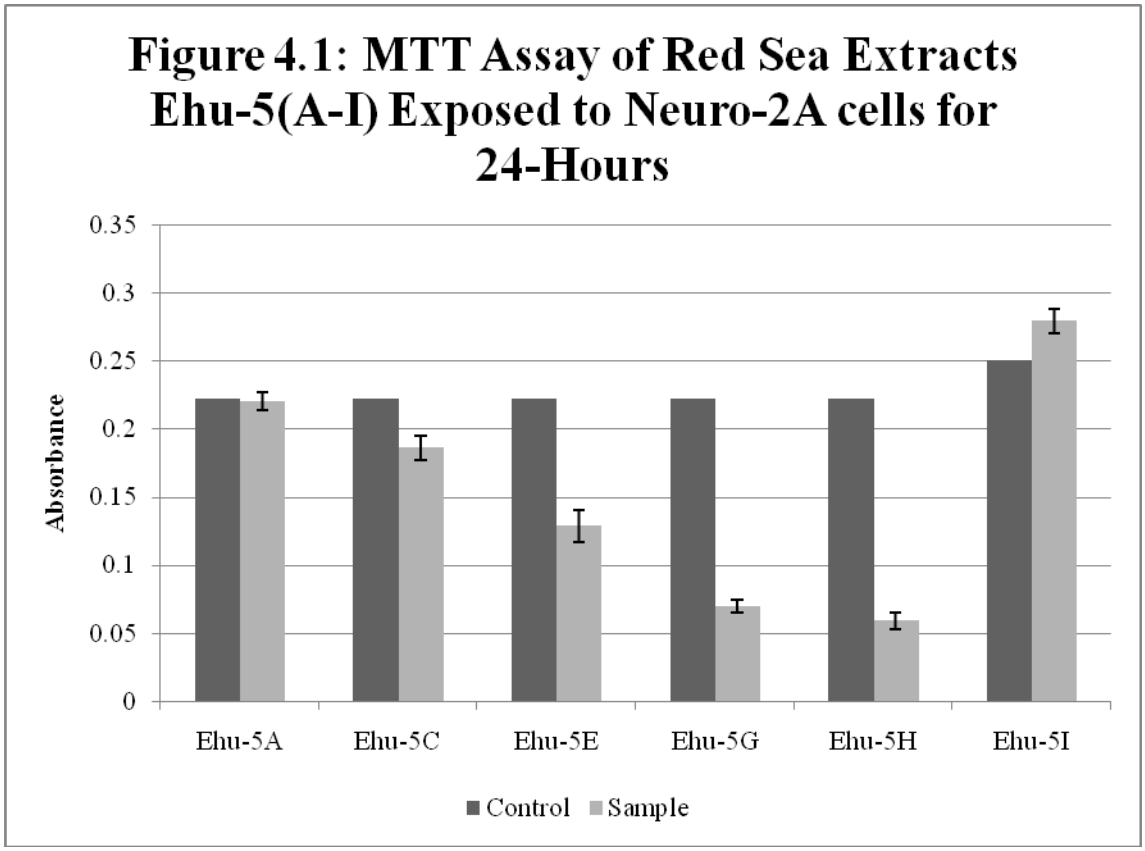


Figure 4.1. Histogram displays absorbance readings for Neuro-2A cells treated with fractionations of Ehu-5 samples for 24 hours with an MTT assay, (Table 4.1). Controls for each sample were completely untreated and cultured on the same plate. Error bars represent the standard error of the mean absorbance.

Table 5.1: MTT Assay of Red Sea Extracts Ehu-5H-(1-5) Exposed to Neuro-2A cells for 24 hours						
Sample Code	**Absorbance Average**	**Standard Deviation**	**Trials**	**Standard Error**	**Control Average**	**Cell Viability**
Ehu-5H	0.059	0.025	4	0.00625	0.22	26.61%
Ehu-5H-1	0.105	0.017	4	0.00425	0.25	41.84%
Ehu-5H-2	0.052	0.01	4	0.0025	0.25	20.82%
Ehu-5H-3	0.018	0.007	4	0.00175	0.25	7.21%
Ehu-5H-4	0.157	0.053	4	0.01325	0.25	62.86%
Ehu-5H-5	0.261	0.02	4	0.005	0.26	100.58%

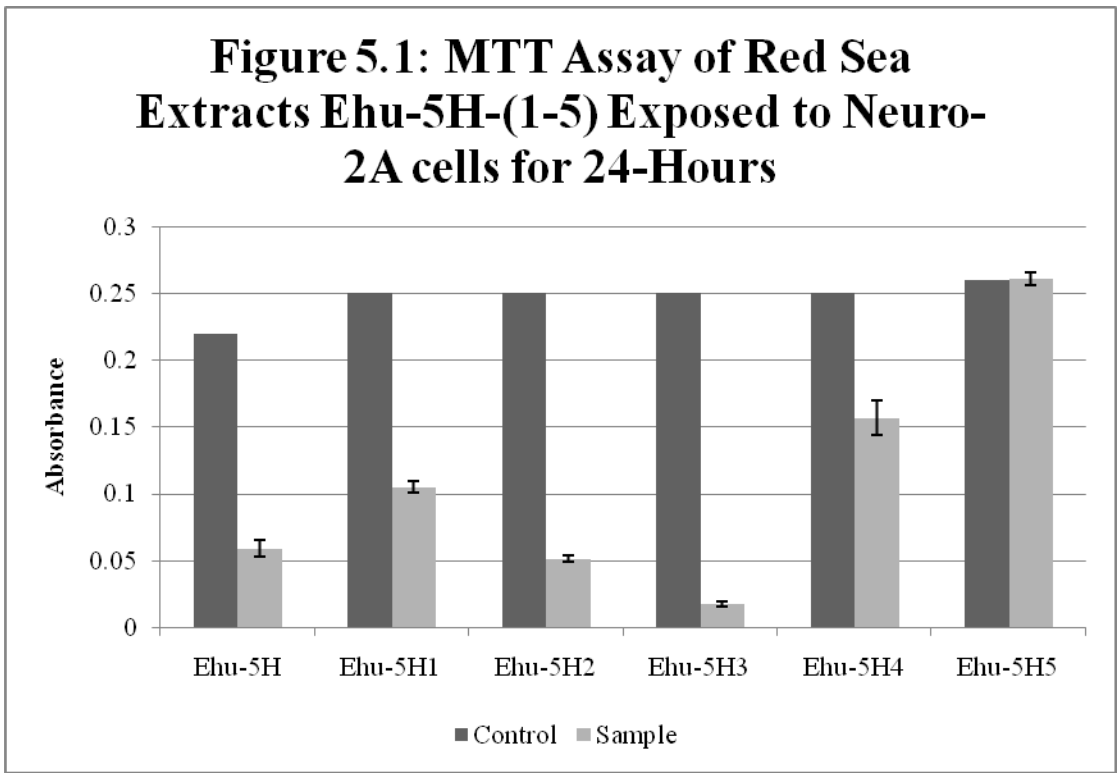


Figure 5.1. Histogram displays absorbance readings for Neuro-2A cells treated with fractionations of Ehu-5H samples for 24 hours with an MTT assay, (Table 5.1). Controls for each sample were completely untreated and cultured on the same plate. Error bars represent the standard error of the mean absorbance.

Figure 5.2: MTT Assay Summary of Ehu-5 Family

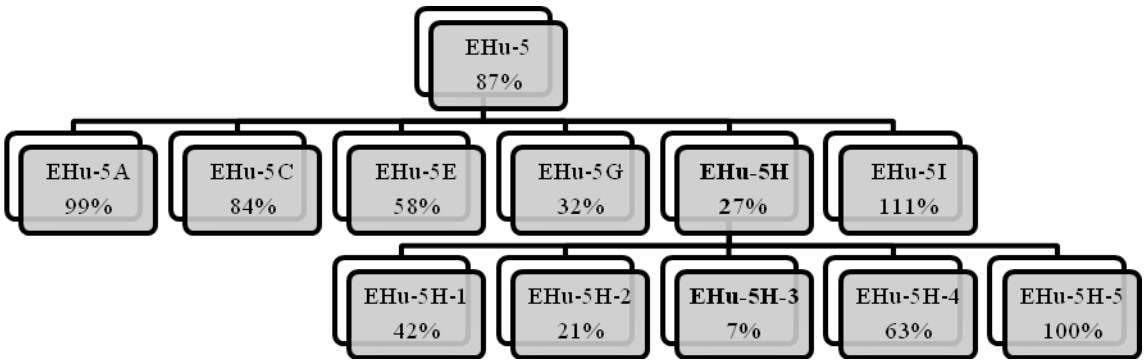


Figure 5.2. Flow chart displays the MTT results for cytotoxic activity for the entire Ehu-5 family. Activity is followed from the parent Ehu-5 to Ehu-5H and Ehu-5H-3, displaying that Ehu-5H-3 is the most active portion of the large parent compound, Ehu-5.

Table 6.1 MTT Assay of Red Sea Extracts Ehu-4H-4(A-G) and Ehu-4G-4 Exposed to Neuro-2A cells for 24 hours						
Sample Code	**Absorbance Average**	**Standard Deviation**	**Trials**	**Standard Error**	**Control Absorbance**	**Cell Viability**
Ehu-4G-4	0.167	0.027	4	0.00675	0.224	74.44%
Ehu-4H-4	0.177	0.019	4	0.00475	0.224	78.79%
Ehu-4H-4A	0.258	0.022	4	0.0055	0.224	114.96%
Ehu-4H-4B	0.055	0.075	4	0.01875	0.224	24.33%
Ehu-4H-4C	0.191	0.018	4	0.0045	0.23	83.04%
Ehu-4H-4D	0.222	0.022	4	0.0055	0.23	96.41%
Ehu-4H-4E	0.193	0.027	4	0.00675	0.23	84.02%
Ehu-4H-4F	0.260	0.025	4	0.00625	0.23	112.93%
Ehu-4H-4G	0.253	0.037	4	0.00925	0.23	109.89%

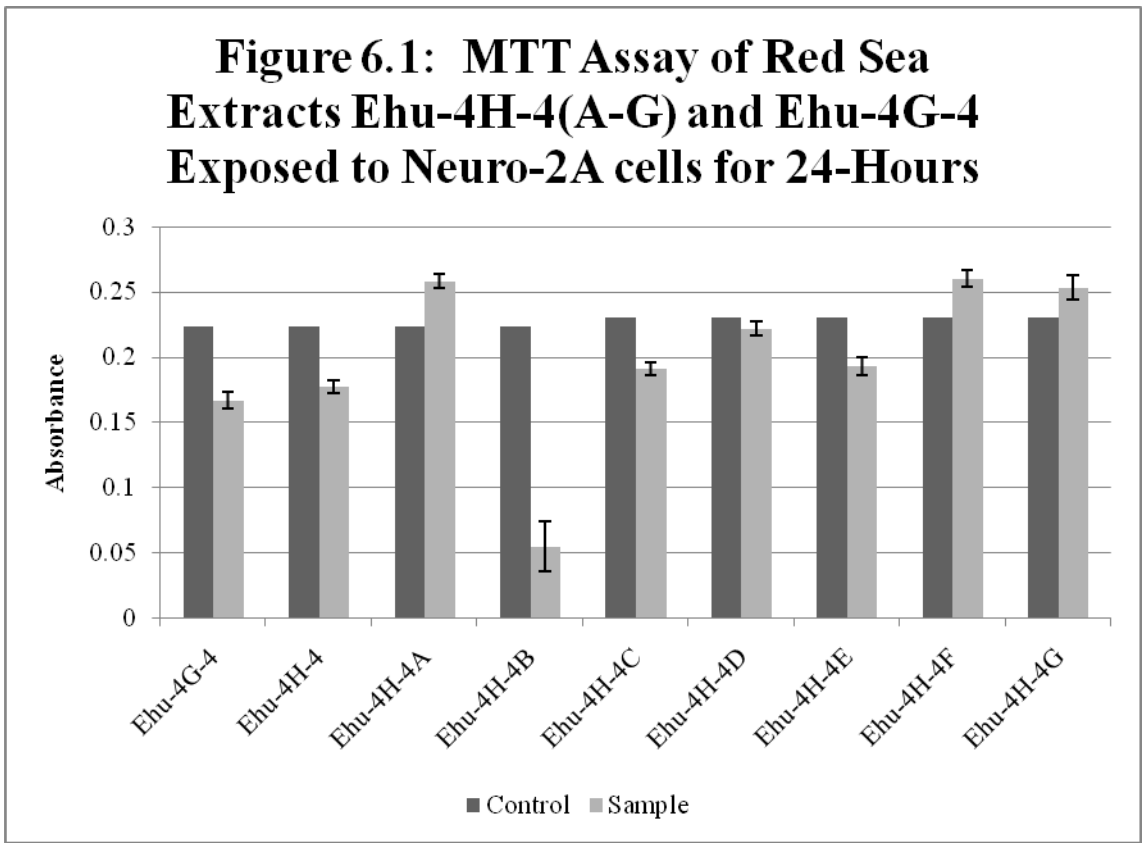


Figure 6.1. Histogram displays absorbance readings for Neuro-2A cells treated with fractionations of Ehu-4H-4 and Ehu-4G-4 for 24 hours with an MTT assay, (Table 6.1). Controls for each sample were completely untreated and cultured on the same plate. Error bars represent the standard error of the mean absorbance.

Table 7.1 MTT Assay of Red Sea Extracts Ehu-1(A-I) Exposed to Neuro-2A cells for 24 hours						
Sample Code	**Absorbance Average**	**Standard Deviation**	**Trials**	**Standard Error**	**Control Absorbance**	**Cell Viability**
Ehu-1A	0.234	0.015	4	0.00375	0.265	88.20%
Ehu-1C	0.238	0.023	4	0.00575	0.265	89.54%
EHu-1E	0.210	0.025	4	0.00625	0.265	79.08%
Ehu-1G	0.231	0.041	4	0.01025	0.265	86.90%
EHu-1H	0.192	0.005	4	0.00125	0.265	72.48%
EHu-1I	0.235	0.017	4	0.00425	0.224	105.02%

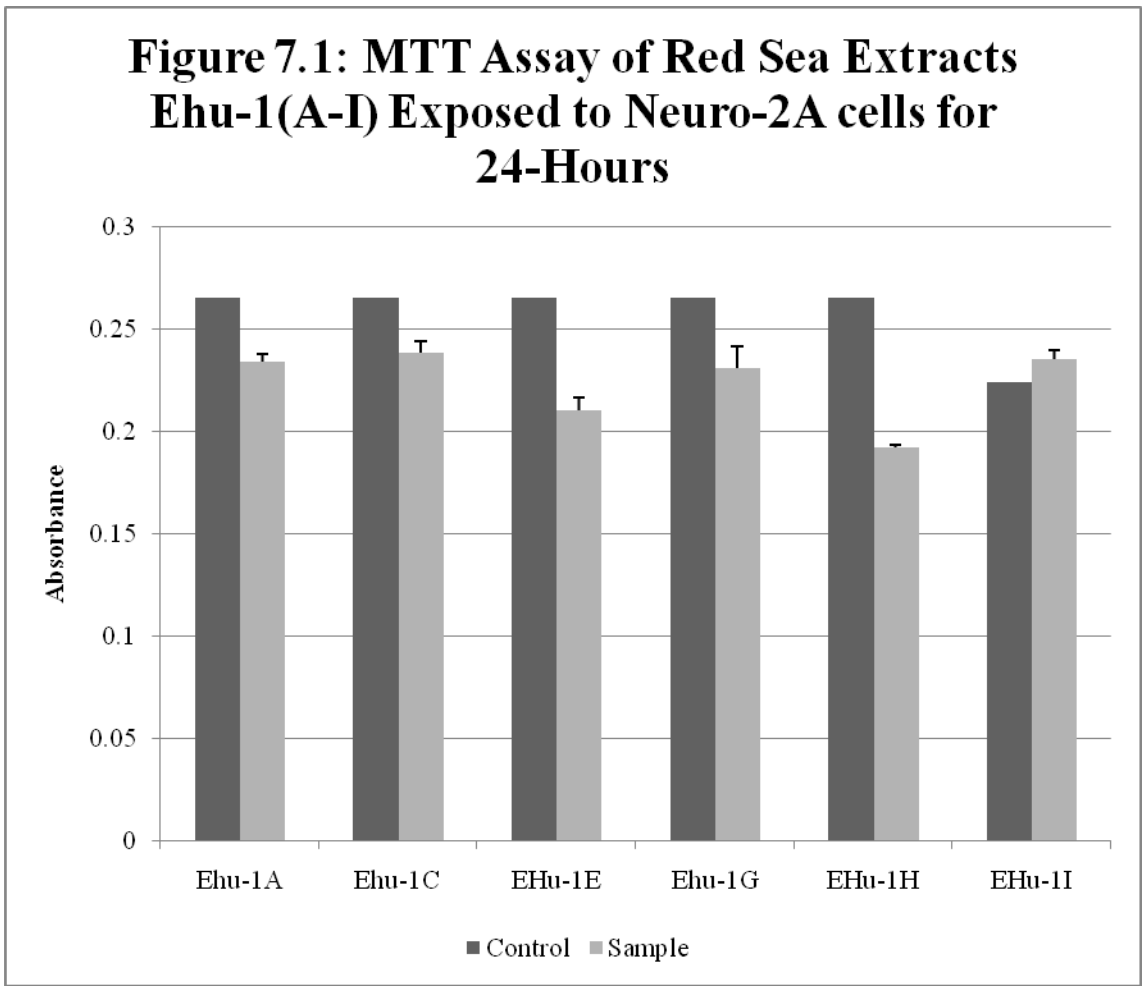


Figure 7.1. Histogram displays absorbance readings for Neuro-2A cells treated with fractionations of Ehu-1 samples for 24 hours with an MTT assay, (Table 7.1). Controls for each sample were completely untreated and cultured on the same plate. Error bars represent the standard error of the mean absorbance.

Table 8.1: MTT Assay of South Africa Priority Extracts Exposed to Neuro-2A cells for 24 hours						
Sample Code	**Absorbance Average**	**Standard Deviation**	**Total Trials**	**Standard Error**	**Control**	**Cell Viability**
SAF04-18	0.189	0.046	4	0.0115	0.237	79.54%
SAF04-19	0.148	0.049	4	0.01225	0.237	62.24%
SAF04-23	0.245	0.019	4	0.00475	0.243	100.93%
SAF04-30	0.210	0.019	4	0.00475	0.214	98.36%
SAF04-53	0.201	0.021	4	0.00525	0.186	108.05%
SAF04-55	0.005	0.001	4	0.00025	0.186	2.42%
SAF04-60	0.237	0.016	4	0.004	0.223	106.05%
SAF04-62	0.134	0.022	4	0.0055	0.223	60.13%
SAF04-65	0.279	0.028	4	0.007	0.198	140.78%

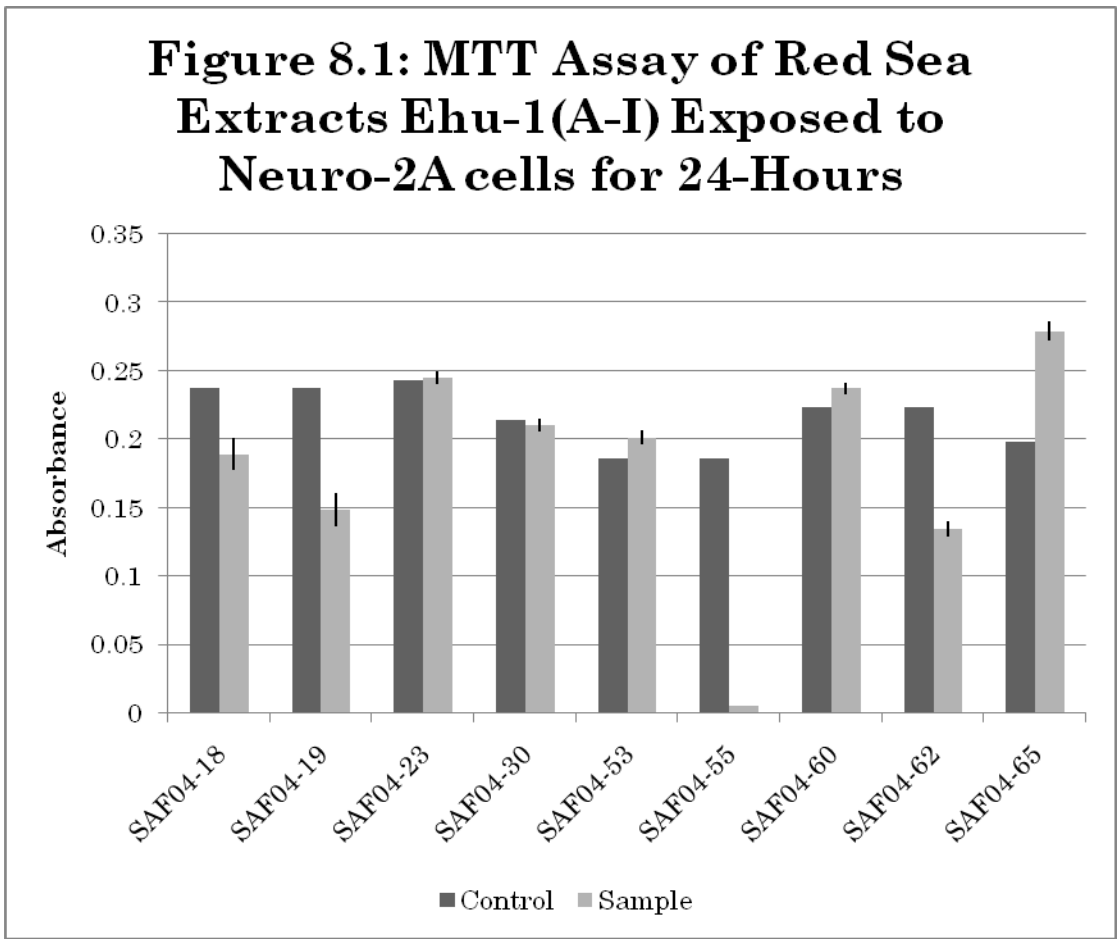


Figure 8.1. Histogram displays absorbance readings for Neuro-2A cells treated with priority SAF04 samples for 24 hours with an MTT assay, (Table 8.1). Controls for each sample were completely untreated and cultured on the same plate. Error bars represent the standard error of the mean absorbance.

Table 9.1 MTT Assay of South Africa Non-Priority Extracts Exposed to Neuro-2A cells for 24 hours						
Sample Code	**Absorbance Average**	**Standard Deviation**	**Total Trials**	**Standard Error**	**Control Absorbance**	**Cell Viability**
SAF04-10	0.174	0.022	4	0.0055	0.191	90.84%
SAF04-11	0.187	0.016	4	0.004	0.191	98.04%
SAF04-12	0.199	0.019	4	0.00475	0.191	103.93%
SAF04-13	0.223	0.031	4	0.00775	0.191	116.75%
SAF04-14	0.184	0.036	4	0.009	0.191	96.07%
SAF04-15	0.228	0.04	4	0.01	0.237	96.20%
SAF04-16	0.209	0.043	4	0.01075	0.237	88.29%
SAF04-17	0.217	0.05	4	0.0125	0.237	91.67%
SAF04-54	0.232	0.03	4	0.0075	0.186	124.30%
SAF04-56	0.215	0.01	4	0.0025	0.186	115.17%
SAF04-57	0.236	0.025	4	0.00625	0.186	126.85%
SAF04-58	0.200	0.027	4	0.00675	0.223	89.59%
SAF04-59	0.188	0.029	4	0.00725	0.223	84.10%
SAF04-61	0.232	0.022	4	0.0055	0.223	103.81%
SAF04-63	0.200	0.027	4	0.00675	0.198	100.76%
SAF04-64	0.206	0.033	4	0.00825	0.198	103.91%
SAF04-66	0.220	0.05	4	0.0125	0.198	110.98%
SAF04-67	0.195	0.057	4	0.01425	0.198	98.48%
SAF04-68	0.212	0.025	4	0.00625	0.236	89.83%
SAF04-69	0.205	0.038	4	0.0095	0.236	86.65%
SAF04-70	0.194	0.012	4	0.003	0.236	82.20%
SAF04-71	0.177	0.023	4	0.00575	0.236	74.89%
SAF04-72	0.202	0.053	4	0.01325	0.236	85.49%

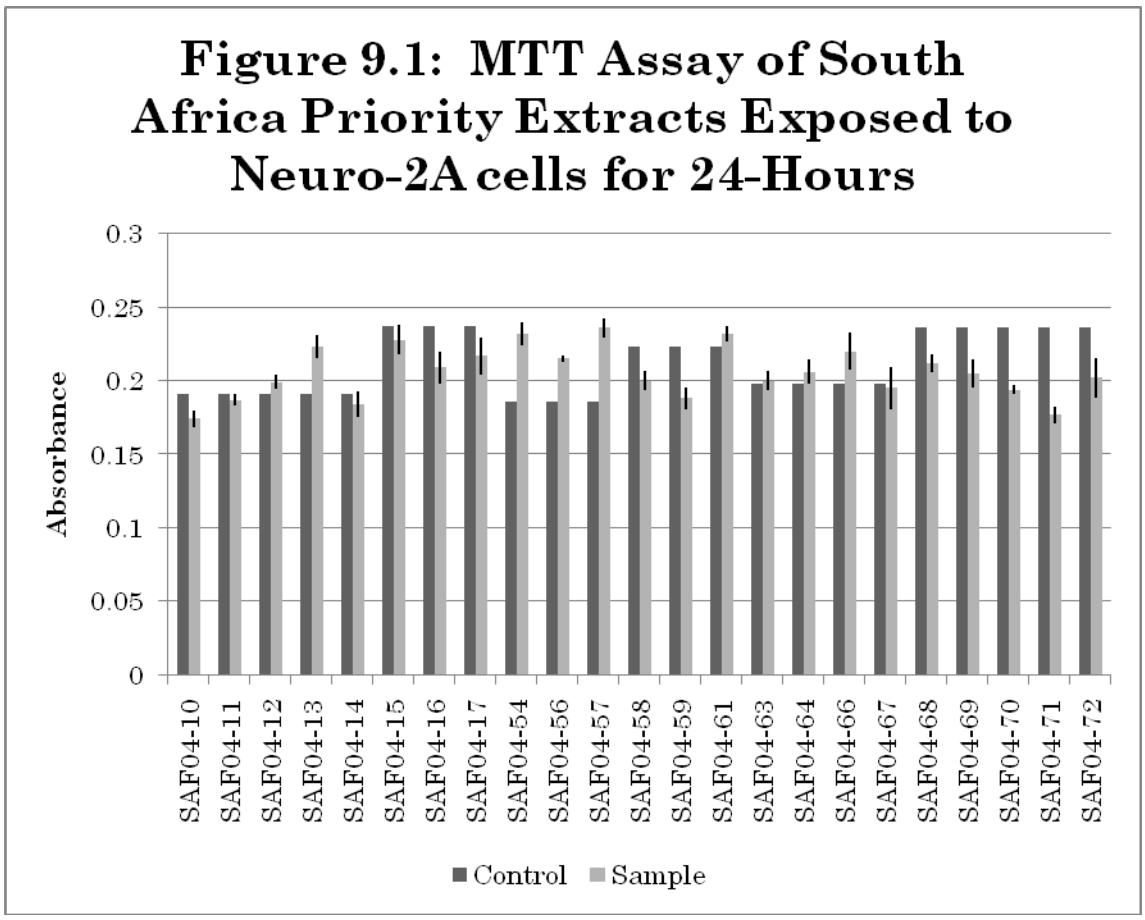


Figure 9.1. Histogram displays absorbance readings for Neuro-2A cells treated with crude non-priority SAF04 samples for 24 hours with an MTT assay, (Table 9.1). Controls for each sample were completely untreated and cultured on the same plate. Error bars represent the standard error of the mean absorbance.

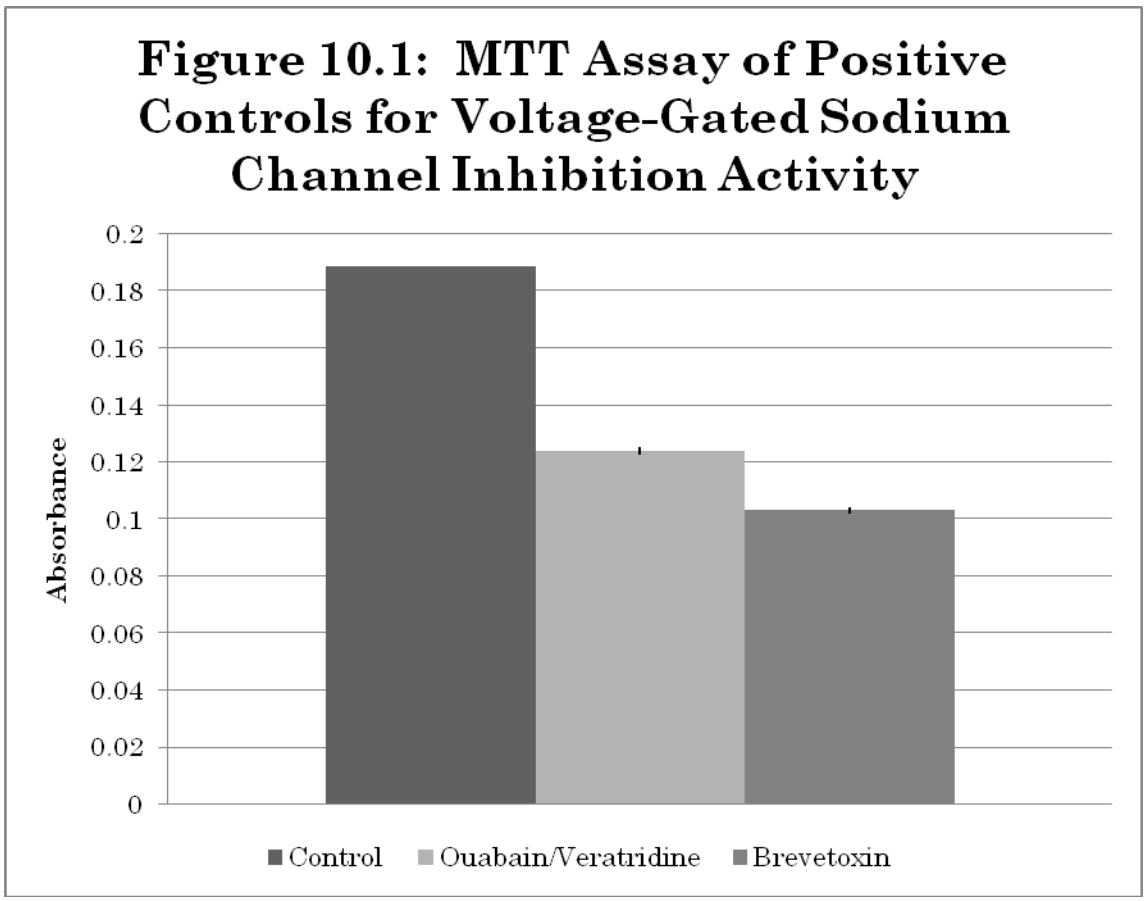


Figure 10.1. Histogram displays absorbance readings for Neuro-2A cells treated with Ouabain/Veratridine control and positive control Brevetoxin for 24 hours (Table 10.1). Error bars represent the standard error of the mean absorbance.

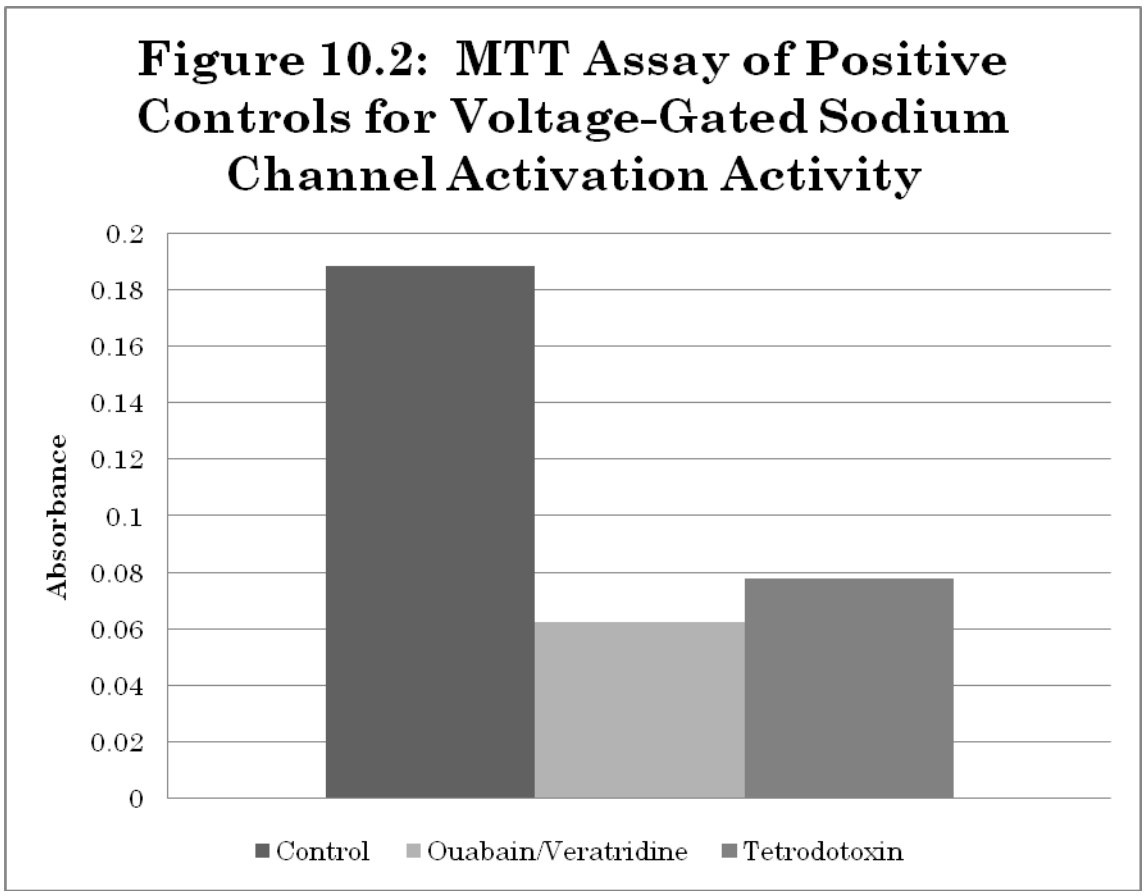


Figure 10.2. Histogram displays absorbance readings for Neuro-2A cells treated with Ouabain/Veratridine control and positive control Tetrodotoxin for 24 hours (Table 10.2). Error bars represent the standard error of the mean absorbance.

Table 10.1: MTT Assay of Positive Controls for Voltage-Gated Sodium Channel Activation Activity on Neuro-2A cells

Control Average	+O/V	Standard Deviation	Standard Error	Cell Viability	O/V +B	Standard Deviation	Standard Error	Cell Viability
0.18875	0.1238	0.023433	0.001464	65.61%	0.1030	0.019433	0.001214	54.61%

Table 10.2: MTT Assay of Positive Controls for Voltage-Gated Sodium Channel Activation Activity on Neuro-2A cells

Control Average	+O/V	Standard Deviation	Standard Error	Cell Viability	+O/V +T	Standard Deviation	Standard Error	Cell Viability
0.18	0.0621	0.0111	0.000693	34.51%	0.0778	0.020633	0.001289	43.24%

Table 11.1: Summary of Cytotoxicity Assay for the 24 Well Plate Format (For 1 Well)			
Step	**Total Volume**	**Extract Concentration**	**DMSO**
Obtain 5 µL of stored extract	5 µL	3µg/µL	100%
Dilute Sample in 20 µL of media to make working sample	25 µL	15µg/25µL =0.6µg/µL	(5µL DMSO/25µL) =20% DMSO
Add 25 µL working sample to each well containing 475 µL media	500 µL	15 µg/500µL =0.03µg/µL =30µg/mL	20%(25µL/500) 1% DMSO

Table 11.2: Summary of Cytotoxicity Assay for the 96 Well Plate Format (For 8 Wells)			
Step	**Total Volume**	**Extract Concentation**	**DMSO**
Obtain 4.5 µL of stored extract	4.5 µL	3µg/µL	100%
Dilute Sample in 22 µL of media to make working sample	26.5 µL	13.5µg/26.5µL =0.509µg/µL	(4.5µL DMSO/26.5µL) =16.98% DMSO
Add 26.5 µL working sample to 423.5 µL media in pipet reservoir. Aliquot 50 µL to each well	450 µL	13.5 µg/450µL =0.03µg/µL =30µg/mL	16.98%(26.5µL/450) 0.999% DMSO

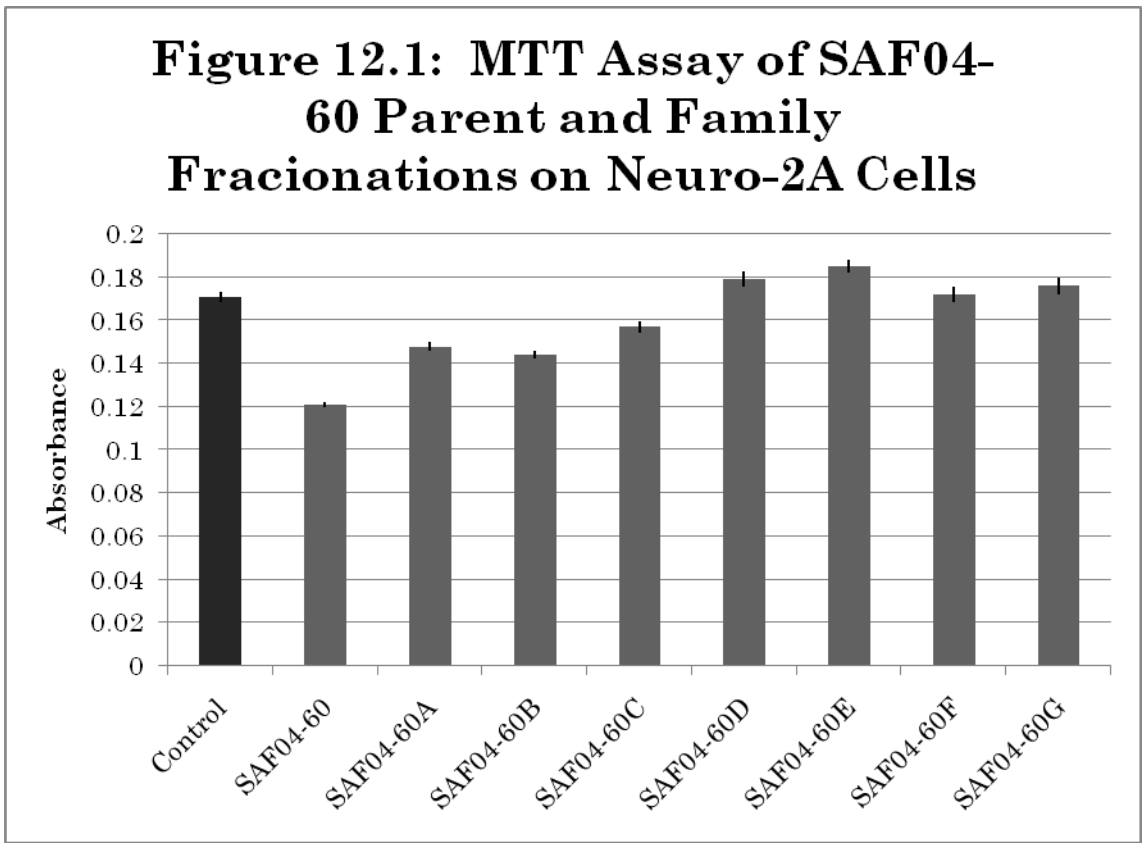


Figure 12.1. Histogram displays absorbance readings for Neuro-2A cells treated with SAF04-60 and its fractionations for 24 hours in the 96 well format (Table 12.1). Error bars represent the standard error of the mean absorbance.

Table 12.1: MTT Assay of South Africa Extract SAF04-60 and Fractionations on Neuro-2A cells for 24 hours					
Sample Code	**Absorbance Average**	**Standard Deviation**	**Total Trials**	**Standard Error**	**Cell Viability**
Control	0.171	0.034	14	0.002428571	
SAF04-60	0.121	0.018	14	0.001285714	72.60%
SAF04-60A	0.148	0.031	14	0.002214286	86.75%
SAF04-60B	0.144	0.026	14	0.001857143	94.07%
SAF04-60C	0.157	0.035	14	0.0025	107.15%
SAF04-60D	0.179	0.05	14	0.003571429	107.15%
SAF04-60E	0.185	0.043	14	0.003071429	111.14%
SAF04-60F	0.172	0.053	14	0.003785714	102.71%
SAF04-60G	0.176	0.054	14	0.003857143	105.34%

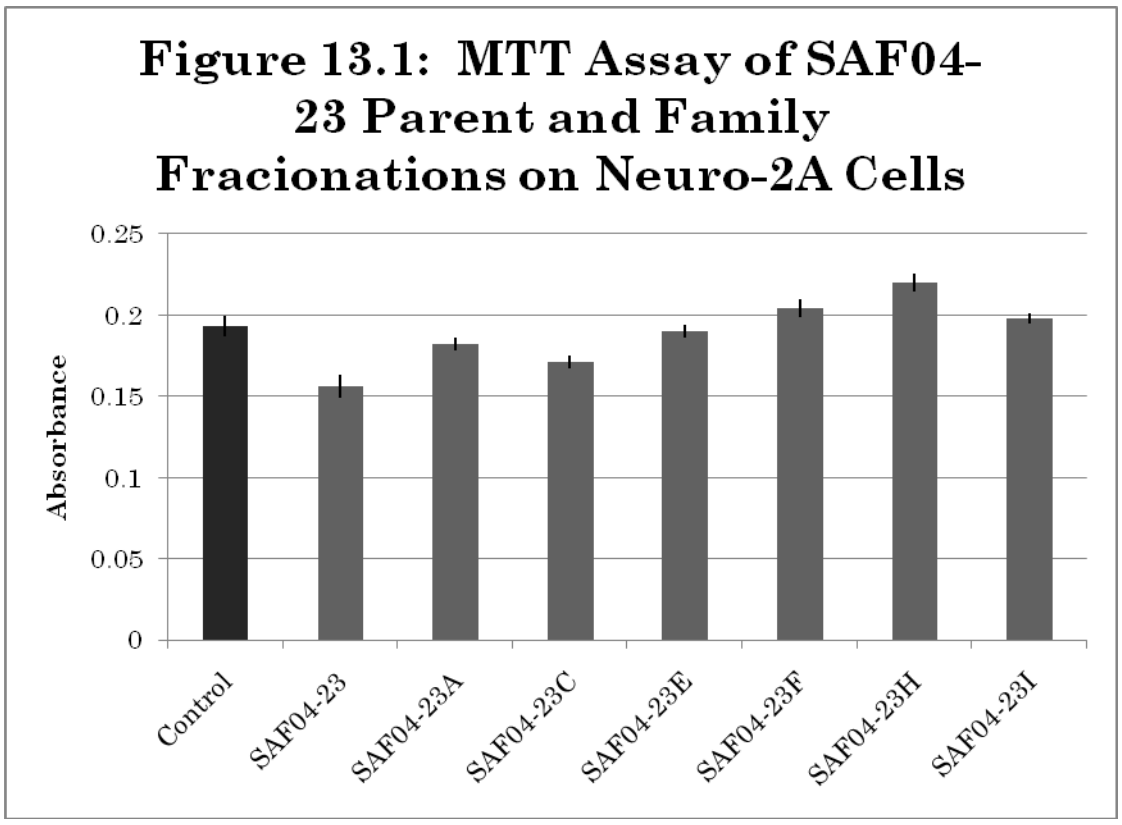


Figure 13.1. Histogram displays absorbance readings for Neuro-2A cells treated with SAF04-23 and its fractionations for 24 hours in the 96 well format (Table 13.1). Error bars represent the standard error of the mean absorbance.

Table 13.1: MTT Assay of South Africa Extract SAF04-23 and Fractionations on Neuro-2A cells for 24 hours

Sample Code	Absorbance Average	Standard Deviation	Total Trials	Standard Error	Cell Viability
Control	0.193	0.039	6	0.0065	
SAF04-23	0.156	0.042	6	0.007	80.50%
SAF04-23A	0.182	0.024	6	0.004	94.13%
SAF04-23C	0.171	0.024	6	0.004	88.27%
SAF04-23E	0.190	0.025	6	0.004166667	98.36%
SAF04-23F	0.204	0.033	6	0.0055	105.52%
SAF04-23H	0.220	0.032	6	0.005333333	113.81%
SAF04-23I	0.198	0.02	6	0.003333333	102.42%

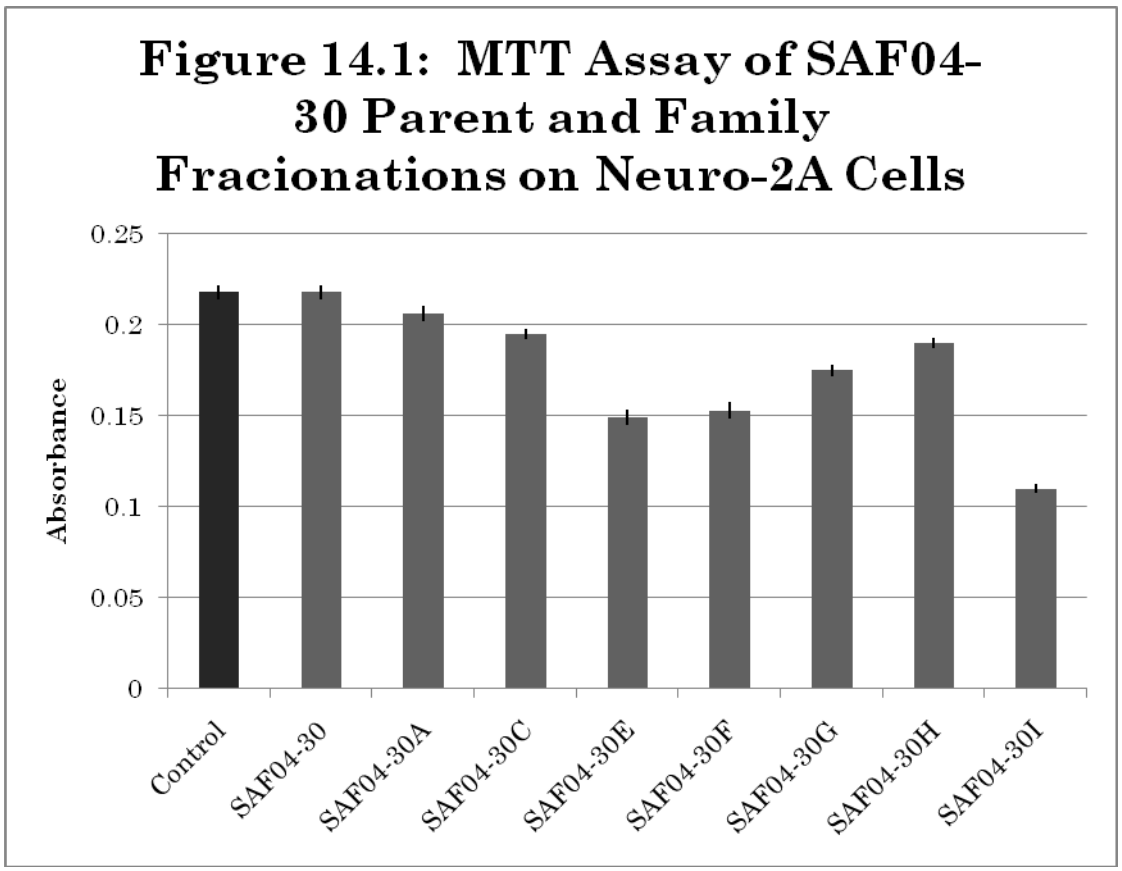


Figure 14.1. Histogram displays absorbance readings for Neuro-2A cells treated with SAF04-30 and its fractionations for 24 hours in the 96 well format (Table 14.1). Error bars represent the standard error of the mean absorbance.

Table 14.1: MTT Assay of South Africa Extract SAF04-30 and Fractionations on Neuro-2A cells for 24 hours					
Sample Code	**Absorbance Average**	**Standard Deviation**	**Total Trials**	**Standard Error**	**Cell Viability**
Control	0.218	0.03	8	0.00375	
SAF04-30	0.218	0.03	8	0.00375	106.95%
SAF04-30A	0.206	0.034	8	0.00425	101.17%
SAF04-30C	0.195	0.022	8	0.00275	95.76%
SAF04-30E	0.149	0.034	8	0.00425	73.45%
SAF04-30F	0.153	0.035	8	0.004375	75.05%
SAF04-30G	0.175	0.027	8	0.003375	85.93%
SAF04-30H	0.190	0.024	8	0.003	93.30%
SAF04-30I	0.110	0.022	8	0.00275	54.03%

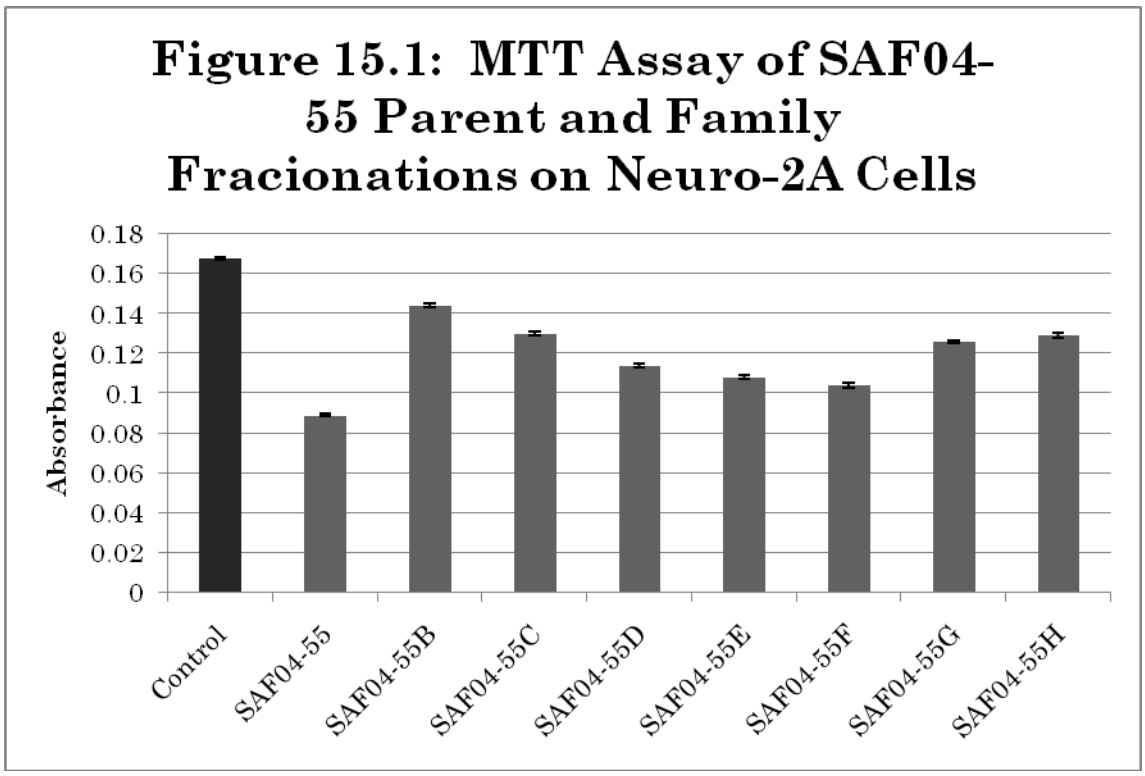


Figure 15.1. Histogram displays absorbance readings for Neuro-2A cells treated with SAF04-55 and its fractionations for 24 hours in the 96 well format (Table 15.1). Error bars represent the standard error of the mean absorbance.

Table 15.1: MTT Assay of South Africa Extract SAF04-55 and Fractionations on Neuro-2A cells for 24 hours					
Sample Code	**Absorbance Average**	**Standard Deviation**	**Total Trials**	**Standard Error**	**Cell Viability**
Control	0.168	0.032	48	0.000666667	
SAF04-55	0.089	0.036	48	0.00075	51.29%
SAF04-55B	0.144	0.034	34	0.001	105.15%
SAF04-55C	0.130	0.035	34	0.001029412	94.89%
SAF04-55D	0.114	0.034	34	0.001	94.42%
SAF04-55E	0.108	0.037	34	0.001088235	88.68%
SAF04-55F	0.104	0.033	34	0.000970588	93.00%
SAF04-55G	0.126	0.028	48	0.000583333	75.81%
SAF04-55H	0.129	0.043	34	0.001264706	102.85%

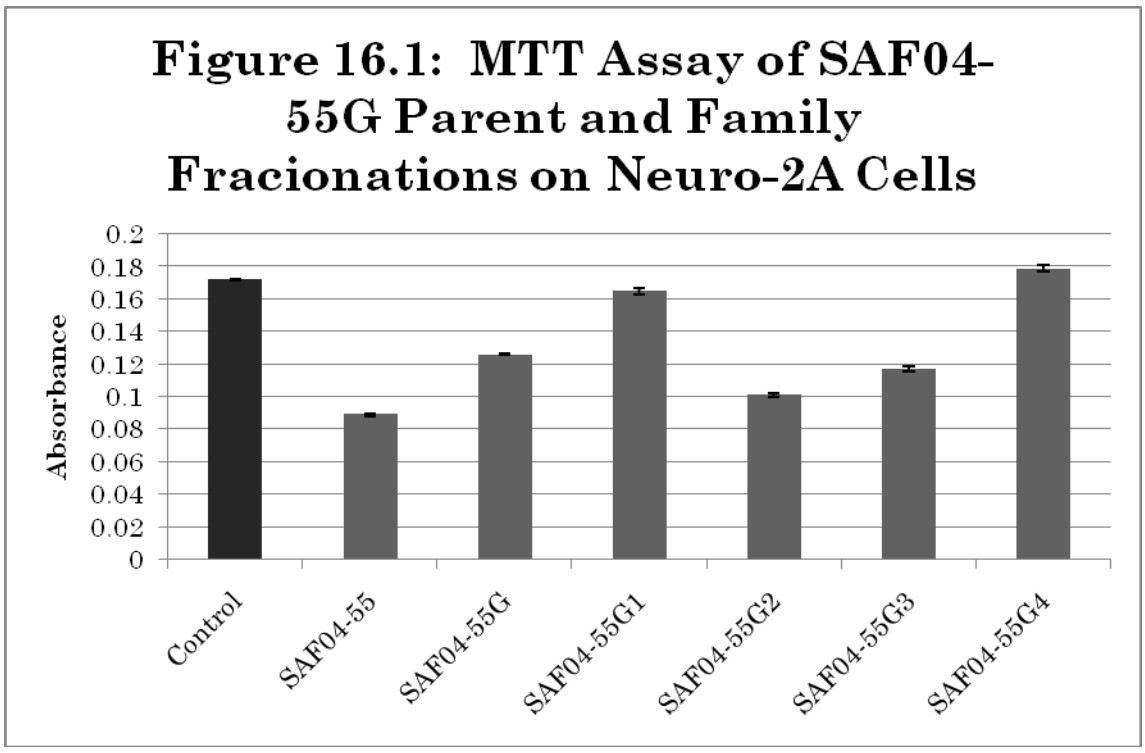


Figure 16.1. Histogram displays absorbance readings for Neuro-2A cells treated with SAF04-55G and its fractionations for 24 hours in the 96 well format (Table 16.1). Error bars represent the standard error of the mean absorbance.

Table 16.1: MTT Assay of South Africa Extract SAF04-55G and Fractionations on Neuro-2A cells for 24 hours					
Sample Code	**Absorbance Average**	**Standard Deviation**	**Total Trials**	**Standard Error**	**Cell Viability**
Control	0.172	0.032	48	0.000666667	
SAF04-55	0.089	0.036	48	0.00075	51.29%
SAF04-55G	0.126	0.028	48	0.000583333	75.81%
SAF04-55G1	0.165	0.041	22	0.001863636	93.32%
SAF04-55G2	0.101	0.032	22	0.001454545	58.33%
SAF04-55G3	0.117	0.037	22	0.001681818	66.44%
SAF04-55G4	0.179	0.037	22	0.001681818	100.69%

Table 17.1: Summary of 24 well results			
Very Slight (85-76%)	**Slight (75-66%)**	**Moderate (65-56%)**	**Strong (Less than 55%**
Ehu-5C (83.99%)	Ehu-4G-4 (74.44%)	Ehu-5E (58.29%)	Ehu-05/27/07 (21.33%)
Ehu-4H-4 (78.79%)	Ehu-1H (72.48%)	Ehu-5H-4 (62.86%)	Ehu-5G (31.68%)
Ehu-4H-4C (83.04%)	SAF04-71 (74.89%)	SAF04-19 (62.24%)	Ehu-5H (26.61%)
Ehu-4H-4E (84.02%)		SAF04-62 (60.13%)	Ehu-5H-1 (41.84%)
Ehu-1E (79.08%)			Ehu-5H-2 (20.82%)
SAF04-18 (79.54%)			Ehu-5H-3 (7.21%)
SAF04-59 (84.10%)			Ehu-4H-4B (24.33%)
SAF04-70 (82.20%)			SAF04-55 (2.42%)

7. References

Alley, M. C., Scudiero, D. A., Monks, A., Hursey, M. L., Czerwinski, M. J., Fine, D. L., et al. (1988). Feasibility of Drug Screening with Panels of Human Tumor Cell Lines using a Microculture Tetrazolium Assay. *Cancer Research , 48* (2), 589-601.

ATCC. (2001). *ATCC Homepage*. Retrieved 2007, from MTT Cell Proliferation Assay Instructions: www.atcc.org

Baden, D. G., Mende, T. J., Szmant, A. M., Trainer, V. L., Edwards, R. A., & Roszell, L. E. (1988). Brevetoxin Binding: Molecular Pharmacology Versus Immunoassay. *Toxicon , 26* (1), 97-103.

Banaja, A. (1990). *Red Sea, Gulf of Aden and Suez Canal : a bibliography on oceanographic and marine environmental research.*

Chang, R. S., Lotti, V. J., Monaghan, R. L., Birnbaum, J., Stapley, E. O., Goetz, M. A., et al. (1985, October 11). A potent nonpeptide cholecystokinin antagonist selective for peripheral tissues isolated from Aspergillus alliaceus. *Science , 230* (4722), pp. 177-179.

Davis, M. H., Pato, C. N., & Gruenstein, E. (1982). Analysis of Neurotoxin and Mitogen-stimulated Sodium Transport in Human Fibroblasts. *The Journal of Biological Chemistry , 257* (8), 4356-4361.

Feher, M., & Schmidt, J. M. (2003). Property Distributions: Differences between Drugs, Natural Products and Molecules from Combinatorial Chemistry. *Journal of Chemical Information and Modeling , 43* (1), 218-227.

Fureman BE, J. H. (2002). Triggers of paroxysmal dyskinesia in the calcium channel mouse mutant tottering. *Pharmacology, Biochemistry and Behavior , 73* (3), 631-637.

Gallacher, S., & Birbeck, T. (1992). A tissue culture assay for direct detection of sodium channel blocking toxins in bacterial culture supernates. *FEMS Microbiology Letters , 92*, 101-108.

Ishiuchi, S., Tsuzuki, K., Yoshida, Y., Yamada, N., Hagimura, N., Okado, H., et al. (2002). Blockage of Ca Permeable AMPA Receptors Suppresses Migration and Induces Apoptosis in Human Glioblastoma Cells. *Nature Medicine , 8*, 971-978.

Kingston-Tivey, M. (1998). The Formation of Mineral Deposits At Mid-Ocean Ridges. *Oceanus , 41* (2), 22-26.

Kogure, K., Tamplin, M. L., Simidu, U., & Colwell, R. R. (1988). A Tissue Culture Assay For Tetrodotoxin, Saxitoxin, and Related Toxins. *Toxicon , 26* (2), 191-197.

Koschinsky, A. G.-S. (2008). Hydrothermal venting at pressure-temperature conditions above the critical point of seawater, 5°S on the Mid-Atlantic Ridge. *Geology , 36*, 615-618.

Levy, S. B. (1994, October 1). Drug Resistance: The New Apocalypse. *Trends in Microbiology , 2* (10), pp. 341-425.

Lieske, E. a. (2004). *Coral reef guide*. London: HarperCollins.

Lindvall, O., & Kokaia, Z. (2006). Stem Cells for the Treatment of Neurological Disorders. *Nature , 441*, 1094-1096.

Manger, R. L., Leja, L. S., Lee, S. Y., Hungerford, J. M., Kirkpatrick, M. A., Yasumoto, T., et al. (2003). Detection of Paralytic Shellfish Poison by Rapid Cell Bioassay: Antagonism of Voltage-Gated Sodium Channel Active Toxins in vitro. *Journal of AOAC International , 86* (3), 540-543.

Medina, R. A., Goeger, D. G., Hills, P., Mooberry, S. L., Huang, N., Romero, L. I., et al. (2008). Coibamide A, a Potent Antiproliferative Cyclic Depsipeptide from the Panamanian Marine Cyanobacterium Leptolyngbya sp. *Journal of The American Chemical Society , 130* (20), 6324-6325.

Mitsiades, C. S., Ocio, E. M., Pandiella, A., Maiso, P., Gajate, C., Garayoa, M., et al. (2008). Aplidin, a Marine Organism-Derived Compound with Potent Antimyeloma Activity In vitro and In vivo. *Cancer Research , 68*, 5216-5225.

Mossman, T. (1983). Rapid Colorimetric Assay for Cellular Growth and Survival: Application to Proliferation and Cytotoxicity Assays. *Journal of Immunological Methods , 65*, 55-63.

Moyes, C. D., & Schulte, P. M. (2005). *Principles of Animal Physiology*. Benjamin Cummings.

National Library of Medicine. (2009, June 10). Retrieved November 19, 2009, from Neurologic Diseases: http://www.nlm.nih.gov/medlineplus/neurologicdiseases.html

Newman, D. J., & Cragg, G. M. (2006). Natural Products as Sources of New Drugs over the Last 25 Years. *Journal of Natural Products , 70*, 461-477.

Pomati, F., Rossetti, C., Calamari, D., & Neilan, B. A. (2003). Effects of Saxitoxin (STX) and Veratridine on Bacterial Na-K Fluxes: a Prokaryote-Based STX Bioassay. *Applied and Environmental Microbiology , 69* (12), 7371-7376.

Simmons, L. T., Andrianasolo, E., McPhail, K., & Gerwick, W. H. (2005). Marine natural products as anticancer drugs. *Molecular Cancer Therapeutics , 4* (2), 333-342.

Taylor, M. W., Radax, R., Steger, D., & Wagner, M. (2007). Sponge-Associated Microorganisms: Evolution, Ecology and Biotechnological Potential. *Microbiology and Molecular Biology Reviews , 71* (2), 295-347.

Thiele, C. J. (1998). Neuroblastoma Cell Lines. *Human Cell Culture , 1*, 21-53.

Van Dover, C. L., German, C. R., Speer, K. G., Parson, L. M., & Vrijenhoek, R. C. (2002, Februrary 15). Evolution and Biogeography of Deep-Sea Vent and Seep Invertebrates. *Science* , 1253-1257.

Wainwright, M., & Swan, H. T. (1986). C.G. PAINE AND THE EARLIEST SURVIVING CLINICAL RECORDS OF PENICILLIN THERAPY. *Medical History , 30*, 42-56.

Yannick, J., Romey, G., & Lazdunski, M. (1980). Toxin-Induced K Efflux through the Na Channel of Neuroblastoma Cells. *Centre de Biochimie du Centre National de la Recherche Scientifique , 111*, 265-273.

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Jay Malmo Date