High Throughput Screening of Extracts From Plants Used in Traditional Chinese Medicine Against *Trypanosoma brucei brucei 427*

by

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A Thesis Proposed to the Graduate Faculty of
Middle Tennessee State University
In Partial Fulfillment of the Requirements
For the Degree of
Master of Science in Biology

Middle Tennessee State University
May 2013

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DEDICATION

This research is dedicated to my mother and father. I love you both.

ACKNOWLEDGEMENTS

I would like to first thank Dr. Anthony Newsome, my major professor, for the help, advice and encouragement he has given me during my time at Middle Tennessee State University. Thanks go also to Jeannie Stubblefield for her assistance with this project both inside and outside of the lab. I would like to acknowledge my committee members Dr. John Dubois and Dr. Gore Ervin for their willingness to serve on my committee and review my thesis. Dr. James Morris of Clemson University was very helpful and provided us with the trypanosomes and offered his advice on how to keep them in culture. To Tana Bowling and Scynexis, I would like to thank you for your advice on how to use high throughput screening techniques for screening trypanosomes. Appreciation is expressed to Dr. Iris Gao for providing me with the plant extracts and permitting me to collaborate with the Tennessee Center for Botanical Medicine Research. To my family, I would like to thank you for all of the words of encouragement and support you have given me over the last two years. Lastly, to everyone else that has helped me achieve success in my research and academics, thank you, as none of this could have been possible without you.

ABSTRACT

Trypanosoma brucei, the protozoan blood parasite, is the etiological agent for African trypanosomiasis (also known as “sleeping sickness”). This parasite is estimated to infect almost 30,000 people each year in central Africa, and has a 100% mortality rate if left untreated. The current drugs used to treat infection by *T. brucei* were developed almost a century ago. These compounds are toxic, expensive, and are becoming ineffective due to increased resistance. There are significant needs for new drug therapies for the treatment of *T. brucei* infections. The purpose of this study was to screen plants used in traditional Chinese medicine for activity against *T. brucei*. A library of 144 crude extracts from 34 different plants was screened against the trypanosomes using high throughput screening techniques and a resazurin based PrestoBlue assay. The extract cytotoxicity was also evaluated using L6 rat skeletal myoblast cells. The chloroform and water extracts of *Scutellaria baicalensis* showed the highest activity against the trypanosomes (IC50 of 11.43 and 19.86 μg/ml) as well as selectivity for the trypanosomes over the mammalian cells. The petroleum ether extracts of *Psychotria rubra*, and *Elephantopus scaber,* the ethyl acetate extract of *Pandanus tectorius*, and the extract of *Belamcanda chinensis* prepared with 95% ethanol all showed promising activity (IC50 of 38.02 – 49.6 μg/ml) and high selectivity. The results suggest that plants used in traditional Chinese medicine may have biochemical compounds of potential interest in the search for better drugs to treat African sleeping sickness.

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Chapter I

Introduction

Introduction

Trypanosoma brucei is a protozoan parasite of humans and animals found in Africa. The genus *Trypanosoma* is included in a group known as the hemoflagellates (Schuster and Sullivan, 2002). Hemoflagellates are blood and tissue parasites whose lifecycle involves both vertebrate and invertebrate (insect) hosts (Schuster and Sullivan, 2002). The invertebrate host typically becomes infected while taking a blood meal from an infected animal (Schuster and Sullivan, 2002). Hemoflagellates, along with other members of the order Kinetoplastida, have an organelle associated with the mitochondria known as a kinetoplast (Schuster and Sullivan, 2002). The kinetoplast contains highly condensed DNA, and is located in the posterior portion of *T. brucei* (Schuster and Sullivan, 2002). *Trypanosoma brucei* is transferred between hosts via the tsetse fly, though only a small number of flies are infected (Walochnik and Aspoek, 2010). People infected with the parasite will eventually develop Human African Trypanosomiasis (HAT) (Urech et al., 2011). This disease is commonly referred to as African sleeping sickness (Urech et al., 2011). Human African Trypanosomiasis is one of 13 diseases known as a neglected tropical disease, with most of the diseases on this list occurring in rural, low-income areas where the inhabitants may be living on as little as $2 a day (Hotez et al., 2007). The onset of sleeping sickness coincides with the parasite leaving the bloodstream and entering the central nervous system (CNS), and it will be fatal if left untreated (WHO, 2012b). Trypanosome infections are believed to have plagued Africa for centuries, with the first written record coming from the historian Ibn Khaldun in

1373 when he reported the death of King Diata II (WHO, 2005). Khaldun reported the king suffered from lethargy and was quoted as saying it was “a disease that frequently befalls the inhabitants” (WHO, 2005). In 1803 Dr. Thomas Winterbottom noticed swollen cervical lymph nodes in slaves who had the disease, and this became a visual diagnostic tool for determining identifying trypanosome-infected patients (WHO, 2005; Ormerod, 1991). In 1901, Emile Brumpt made the link between the tsetse fly and sleeping sickness for the first time, by realizing that the distribution of disease correlated with the distribution of the fly (WHO, 2005). While it was hypothesized that the tsetse fly bite was causing the disease, the first trypanosome infection of humans observed in a blood sample was in 1901 by members of the Liverpool School of Tropical Medicine. It was named *Trypanosoma gambiense*, but the link between the organism and the disease was not made (WHO, 2005). Aldo Castellani, an Italian bacteriologist, using a new method to examine cerebrospinal fluid (CSF), discovered trypanosomes in 59% of patients with sleeping sickness and proposed it to be the etiological agent reported in 1903(Castellani, 1903). David Bruce, a man who discovered trypanosomiasis in cattle and identified the vector, tested Castellani’s hypothesis, and found that sleeping sickness was caused by the trypanosomes seen by Castellani and were transmitted by *Glossina palpalis* (Bruce et al., 1903). Authorities set up infection control operations once a clear cause of disease was established (Simarro et al., 2011).

There are two subspecies of *Trypanosoma brucei* known to cause disease in humans*: Trypanosoma brucei gambiense* and *Trypanosoma brucei rhodesiense* (Barrett et al., 2007). A third subspecies, *Trypanosoma brucei brucei,* is physiologically identical to *T. gambiense and T. rhodesiense,* but cannot cause disease in humans due to susceptibility to a serum high density lipoprotein (Hager, 1994). These subspecies are found in geographically distinct areas and have slight differences in their progression to sleeping sickness (Brun et al., 2010). The geographical distributions of *T. gambiense* and *T. rhodesiense* are shown in Figure 1.

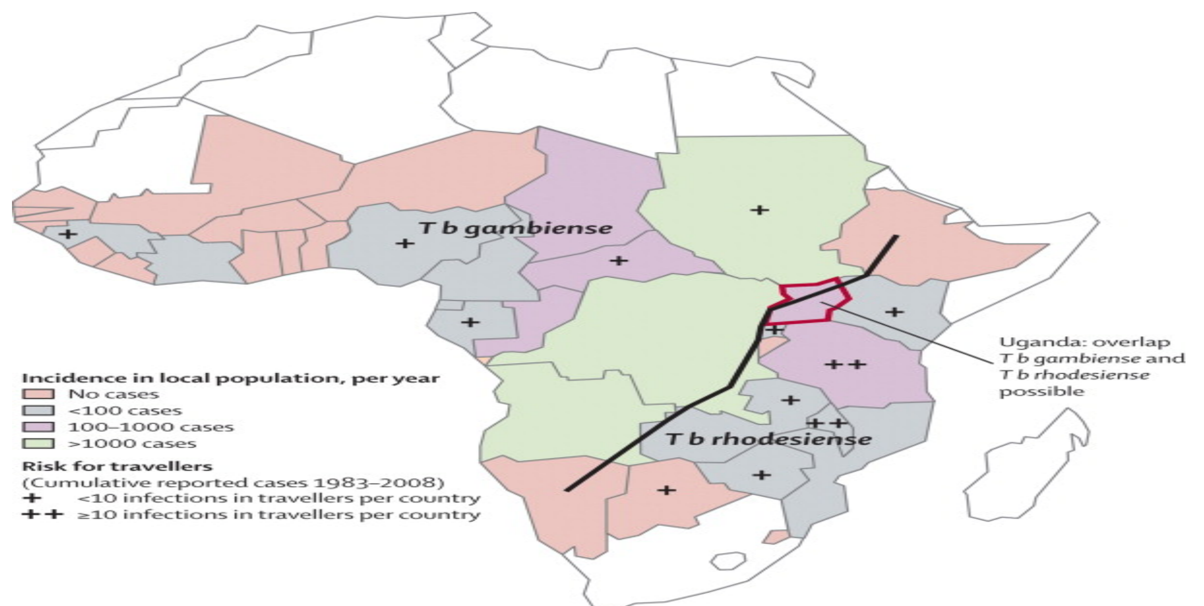


Figure 1. A distribution map showing the occurrence of trypanosome infection in local populations per year (Brun, 2010, with permission)

Trypanosoma rhodesiense is seen in both southern and eastern Africa and causes a quickly progressing variety of trypanosomiasis that is rapidly fatal, with death occurring within weeks to months (Brun et al., 2010). *Trypanosoma gambiense* causes a more chronic, long-term infection, which can take several years to kill the

host if the infection goes untreated (Dumas and Bouteille, 1996). While *T. gambiense* is generally slower to reach CNS involvement than its eastern counterpart, there have been cases of a higher virulence genotype of *T. gambiense* infecting humans and causing an acute onset similar to that of *T. rhodesiense* (Truc et al., 2012b). Both subspecies of *T. brucei* are present in Uganda, and an overlap is possible, with *T. rhodesiense* has a reservoir in the livestock of Uganda (Kuepfer et al., 2011; Wardrop et al., 2010).

Trypanosoma brucei has a complicated lifecycle that involves an invertebrate intermediate host, the tsetse fly, as well as several forms of the parasite (Zikova et al., 2009). Transmission via the bite of a tsetse fly is the most common way the parasite is transmitted, but infection has also occurred due to the parasite crossing the placenta, as well as accidental infections in laboratory settings due to penetration of the skin by contaminated needles (Fevre et al., 2006; WHO, 2012b). When a blood meal is taken from an infected animal by the tsetse fly, the blood stream trypomastigote (of which there are both long and slender trypomastigotes and short and thick trypomastigotes) is ingested (Roditi and Lehane, 2008). The slender trypomastigotes are more susceptible to proteases in the fly's midgut and are probably killed (Roditi and Lehane, 2008). The shorter trypomastigotes survive and differentiate into a procyclic form more suited to living in the fly, and begin to multiply via binary fission (Roditi and Lehane, 2008). These procyclic trypomastigotes become epimastigotes and migrate to the tsetse fly's salivary glands, where they multiply and transform to the infective metacyclic

trypomastigote stage (Roditi and Lehane, 2008). These metacyclic forms enter the mammalian host when the infected fly takes a blood meal (Brun et al., 2010). Once injected into the host they enter the lymphatic system, and eventually pass into the bloodstream where they become bloodstream trypomastigotes and replicate via binary fission (CDC, 2010). The infection will eventually enter the central nervous system of the host, leading to the second stage of infection and the neurological symptoms of sleeping sickness (Barrett, 1999). The lifecycle of *T. brucei* is outlined in Figure 2.

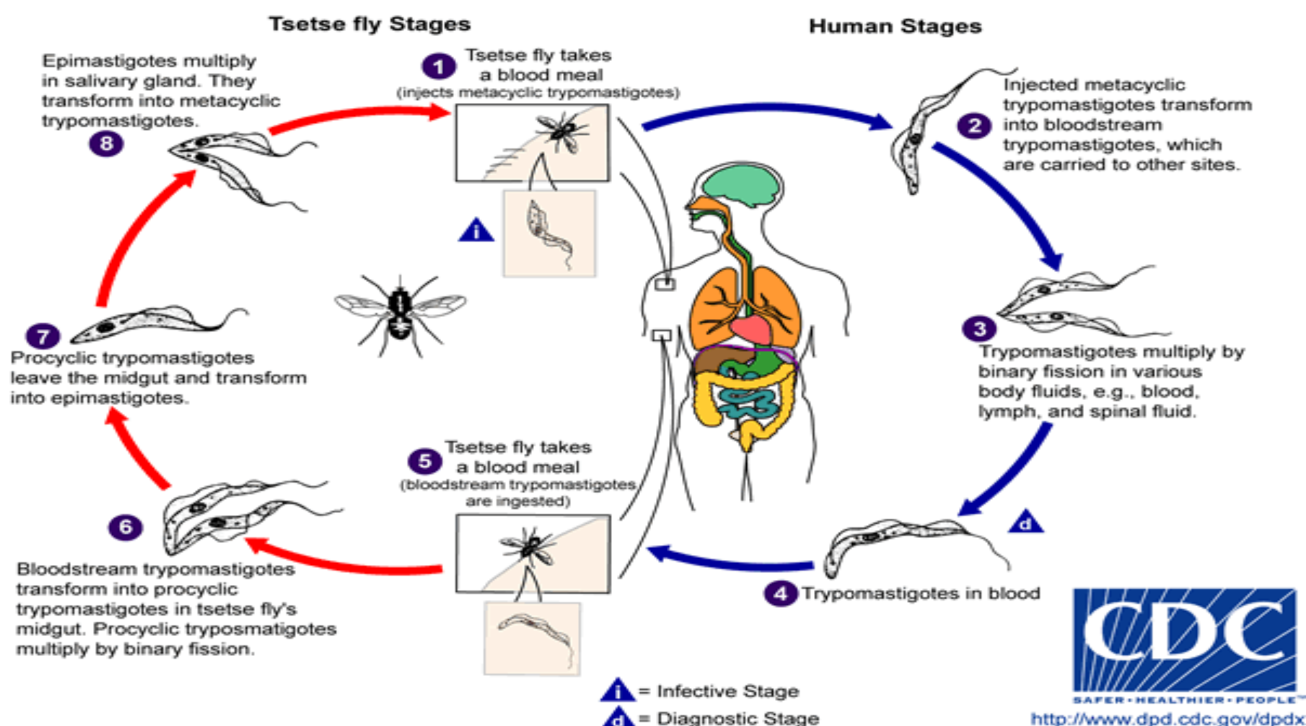


Figure 2. *Trypanosoma brucei* life cycle (from CDC website http://www.cdc.gov/parasites/sleepingsickness/biology.html with permission)

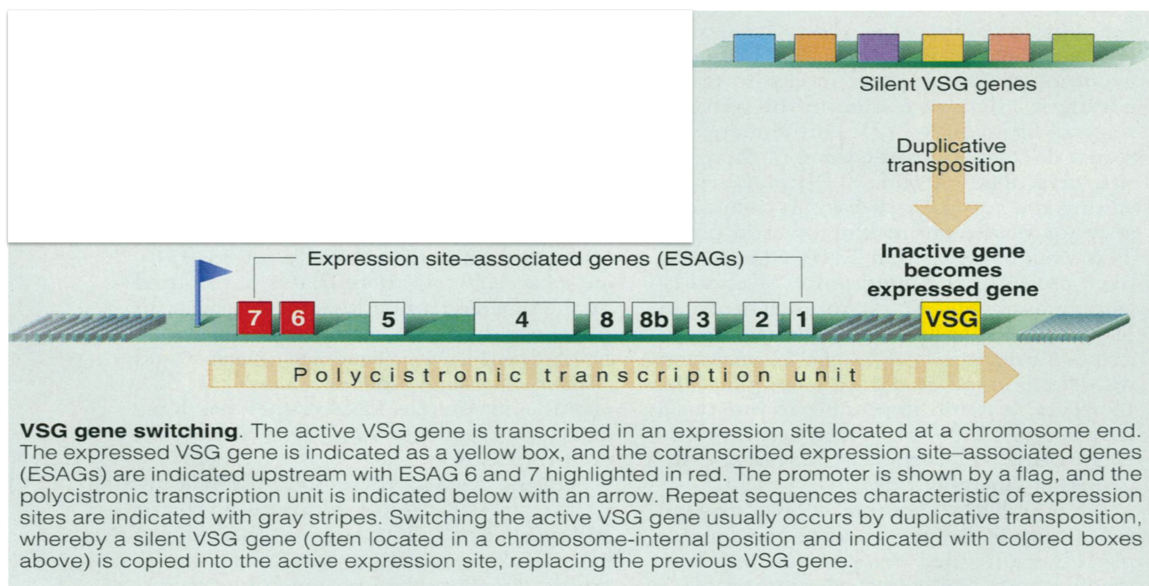
Infection with *T. brucei* begins when the tsetse fly injects the organism into the subcutaneous tissue, leading to a chancre due to a localized inflammatory response (Fevre et al., 2006). Subsequent disease development has two stages, the

first stage is known as the haemolymphatic stage (blood/lymph stage) and the second stage is known as the meningoencephalitic (CNS involvement) stage (Brun et al., 2010). An intermittent fever is seen with the initial stage but is rare in the second stage (Brun et al., 2010). Generally, the symptoms related to the initial stage are headache, itching, and lymphadenopathy, and swollen neck glands commonly referred to as Winterbottom's sign (Brun et al., 2010; Ormerod, 1991). The blood-borne parasites will eventually cross the blood-brain barrier. How the trypanosomes accomplish this is unknown, but recent research suggests that production of Ca^{2+} ions by parasite cysteine proteases induces a signal pathway in the endothelium (Nikolskaia, 2006). This pathway results in the cells becoming permissible to traversal by the trypanosome while maintaining their structural integrity (Nikolskaia, 2006). Once the trypomastigote penetrates the blood-brain barrier, it begins multiplying in the CNS. This causes the neurological symptoms, with the sleep disorder being the most notable and where the disease recieved its name (Brun et al., 2010). The disease disrupts the circadian rhythm, and the sleep cycle becomes fragmented (Brun et al., 2010). The exact mode of action *T. brucei* uses to disrupt the circadian rhythm is unclear, however research has shown it has an affect on the input pathway, oscillator, and output pathway of the circadian system (Lundkvist, 2004). Trypanosome infection affects the input system by causing a decrease in glutamate receptor expression in the suprachiasmatic nuclei (SCN) of the hypothalamus (Lundkvist, 2004). These receptors are key to the body's response to light and dark. Trypanosomes also have been shown to cause a decrease

in the oscillating spontaneous firing of neurons in the SCN (Lundkvist, 2004). Output pathway effects include having decreased melatonin production, decreased body activity, and body temperature rhythm reaching its peak several hours early (Lundkvist, 2004). It has been suggested that these changes are induced by interferon-γ produced in response to the infection (Lundkvist, 2004). Other symptoms of the neurological phase include seizures, impaired motor functions, partial paralysis, and abnormal movements (Brun et al., 2010). Psychiatric symptoms may include aggression, apathy, irritability, and psychosis (Brun et al., 2010). Infection with both subspecies of trypanosomes will progress to the neurological stage and eventually cause coma and/or death without proper treatment (WHO, 2012b).

Once infected with *Trypanosoma brucei,* it is difficult for the immune system to eliminate the parasite because it has developed a method to fool the immune system by changing its surface proteins (Borst and Rudenko, 1994). This also poses a problem for vaccine development because, while an antibody to one antigen will protect against the trypanosomes displaying that protein, it will not provide protection against one displaying a different antigen (Barbet and Mcguire, 1978; La Greca and Magez, 2011). The trypanosome surface is covered with a single protein molecule known as the variable surface glycoprotein (VSG) (La Greca and Magez, 2011). The trypanosomes' genome includes 6 expression regions containing a promoter, expression site-associated genes (ESAG), and an expressed VSG gene (Borst and Rudenko, 1994). An area of the trypanosomes genome encoding a VSG

gene can be seen in Figure 3. The organism has a library of around 10^3 silent VSG genes that it can switch into the expression site by a process called duplicative transposition (Borst and Rudenko, 1994). Variability is created by switching the expression region that is used and by switching the VSG gene itself (Borst and Rudenko, 1994).



VSG gene switching. The active VSG gene is transcribed in an expression site located at a chromosome end. The expressed VSG gene is indicated as a yellow box, and the cotranscribed expression site–associated genes (ESAGs) are indicated upstream with ESAG 6 and 7 highlighted in red. The promoter is shown by a flag, and the polycistronic transcription unit is indicated below with an arrow. Repeat sequences characteristic of expression sites are indicated with gray stripes. Switching the active VSG gene usually occurs by duplicative transposition, whereby a silent VSG gene (often located in a chromosome-internal position and indicated with colored boxes above) is copied into the active expression site, replacing the previous VSG gene.

Figure 3. VSG gene production (Borst and Rudenko, 1994, with permission)

Over the last century there have been several epidemics of HAT throughout Africa. The first epidemic occurred between 1896 and 1906 in Uganda as well as the Congo Basin, followed by a more widely distributed epidemic in 1920 (WHO, 2012b). There were many deaths in the 1920's due to HAT, and in some cases, entire villages were wiped out (Truc et al., 2012a). Dr. Eugene Jamot created mobile teams to combat trypanosome infection (Truc et al., 2012a). The teams were based on three guidelines: examine and treat the highest possible percentage of "at risk" populations, team workers should be specially trained, and services should be autonomous for the patient (WHO, 2012a). Detection of infection by these teams

was performed using palpitation and puncture of glands, and each positive case was treated by atoxylisation, which is the administration of the drug Atoxyl (WHO, 2012a; Louis et al., 2002). This method helped lower HAT occurrence until the 1960's, and almost wiped out the disease (Truc et al., 2012a; WHO, 2012b). The most recent epidemic occurred in 1970 (WHO, 2012b). During these epidemic periods, some affected villages reached greater than a 50% infection rate, at which point sleeping sickness became the first or second leading cause of death in those areas, causing even more death than HIV/AIDS (WHO, 2012b).

Today, there are nearly 60 million people residing in 36 different countries currently susceptible to infection with *T. brucei,* 24 of which are endemic to the *T.b. gambiense* subspecies (Merschjohann and Steverding, 2006; WHO, 2012a). Of the reported cases, 95% were attributed to infection with *T.b. gambiense* (WHO, 2012b). The occurrence of new cases has declined since 2001, but some countries reported nearly 80% of infected individuals were in the meningoencephalitic stage (WHO, 2012a; Truc et al., 2012b). In 2010 there were only 7,139 new cases reported, however, due to the large majority of cases going unreported, the actual estimated number of new cases was closer to 30,000 (WHO, 2012b).

Diagnosing a trypanosome infection is key to beginning treatment. The main method for diagnosing sleeping sickness is to observe the parasites in the blood through microscopy (Fevre et al., 2006). There are also several other methods that can be used. One inexpensive and widely used diagnostic tool is the Card Agglutination Test for Trypanosomiasis (CATT), which costs only $0.40 USD to

perform (Fevre et al., 2006). The CATT tests a patient's serum for antibodies to a prominent trypanosome antigen. However, the test can not differentiate between current and past infections, and there are a small number of trypanosomes that do not express the antigen, so other clinical tests are required for confirmation (Fevre et al., 2006). PCR can be used to detect trypanosome infection, though there have been reports of problems of reproducibility, as well as problems calculating sensitivity attributable to occasional low levels of trypanosomes in the bloodstream during chronic infections (Koffi et al., 2006; Fevre et al., 2006). While serological tests for the parasite are suggestive, identification of the parasite in fluids or tissues is generally needed to begin treatment due to high toxicity of the medicines administered as treatment (Koffi et al., 2006).

Human African trypanosomiasis is treated today using essentially the same drugs that were used seventy to eighty years ago (WHO, 2012b). In the early stages of the disease a patient is treated with either pentamidine or suramin, both of which produce undesirable side effects (WHO, 2012b). Treatment of the second stage of disease requires drugs that can cross the blood-brain barrier and are very toxic and difficult to administer (WHO, 2012b). The meningoencephalitic stage of HAT is treated with melarsoprol, which is derived from arsenic and is fatal to the patient in 3-10% of cases (WHO, 2012b). Stage 2 can also be treated with eflornithine, a drug whose side effects are less severe than melarsoprol, but only works with infections of *T.b. gambiense* and is very difficult to administer (WHO, 2012b). These drugs are not only toxic, but are beginning to lose effectiveness due to spreading resistance

(WHO, 2012; Barrett et al., 2011). Melarsoprol failures were first seen in the 1990s, and their incidence has continued to increase (Barrett et al., 2011). This has been attributed to a loss of a plasma membrane transporter integral to the uptake of both melarsoprol and pentamidine, though failure of pentamidine is less common (Barrett et al., 2011). Eflornithine is beginning to replace melarsoprol as the primary choice for treatment. However, it has been demonstrated that if a certain amino acid transporter key to the uptake of the drug is lost, resistance could occur (Barrett et al., 2011). Due to the toxicity of the drugs used to treat HAT, as well as the concern of growing resistance, new methods of treatment should be pursued.

The use of medicinal plants to treat disease is a widely accepted practice, with 80% of the world's population engaging in some type of traditional medicine use (Abiodun et al., 2012). There are over 250,000 flowering plants with medicinal properties worldwide (Mbaya and Ibrahim, 2011). More than 25% of the world's common drugs contain at least one compound of natural origin, as do 75% of the drugs used to treat cancer (Mann et al., 2011; Newman, 2012). The testing of plants used in traditional Chinese medicine (TCM) for biological activity against infectious agents has recently been shown to be effective, leading to the production of a novel malaria drug, Artemisinia, from the Sweet Wormwood Herb, which was discovered due to the collaborative effort of over 600 Chinese scientists (Cui, 2009). To date, although there are no drugs of plant origin used in the treatment of HAT, plants (such as *Ocimum grattissimum*) exhibit anti-trypanosomal properties in an experimental setting (Abiodun et al., 2012).

The purpose of the current study was to examine extracts from plants used in TCM for the possibility of inhibition of *T. brucei*, with the potential to be used in drug development to treat *T. brucei* infections in humans*.* This will be achieved by performing viability assay (PrestoBlue fluorescence activity) experiments on trypanosomes that have been subjected to extracts from plants used in TCM.

Chapter II

Materials and Methods

Materials and Methods

Plants and Extracts

The plant extracts tested in this study were from plants currently used in traditional Chinese medicine. All extracts were received from the Center for Botanical Medicine Research at Middle Tennessee State University, who obtained them through the Guangxi Medicinal Plants Botanical Garden (GMPBG) in Nanning, Guangxi province, China. The GMPBG is a 202-hectacre garden where medicinal plants are grown and their properties are researched. It is listed as having the largest variety of medical plants in the world. Extract creation was accomplished using several different solvents on ground plant parts. The solvents used were ethanol, petroleum ether, ethyl acetate, butanol, water, and chloroform. Once extracts were obtained the solvents were evaporated, and the remaining material was suspended in dimethyl sulfoxide (DMSO) at a concentration of 10 mg/ml. The extract library consisted of a total of 144 extracts from 34 different plants. A complete list of the plant species, plant parts, solvents used for extraction, and current uses for the plants used in this study are included in Appendix I.

Parasite Culture

The bloodstream form of *Trypanosoma brucei brucei 427* was used in all plant extract sensitivity assays. The trypanosomes were provided by Dr. James Morris of Clemson University (Clemson, SC) and were maintained in HMI-9 media. The HMI-9 media was prepared onsite as described by Hirumi and Hirumi (1989). Each batch of media was prepared with 365ml Iscove's Modified Dulbecco's Medium

(Invitrogen, Carlsbad, CA), 50 ml heat inactivated fetal bovine serum (Atlanta Biologicals, Atlanta, GA), 50 ml serum plus (Sigma, St. Louis, MO), 5 ml of hypoxanthine stock, 26.25 ml of HMI mix, and 5.25 ml of penicillin/streptomycin (GIBCO, Grand Island, New York). Hypoxanthine stock was 13.5 mg/ml of hypoxanthine in NaOH and was prepared by adding 37 ml dH_2O and 37 ml of 2N NaOH with 1g of hypoxanthine in a glass container. The HMI mix was prepared by combining 282 mg of 100X Bathocuproine disulfanic acid salts (Sigma, St. Louis, MO) with 100 ml dH_2O, 390 mg 100X Thymidine (Sigma, St. Louis, MO) with 100 ml dH_2O, 1,100 mg 100X pyruvate (Sigma, St. Louis, MO) with 100 ml dH_2O, 140 μl 100X β-mercaptoethanol (Sigma, St. Louis, MO) with 100 ml dH_2O, and 1820 mg L-cysteine (Sigma, St. Louis, MO) with 100 ml dH_2O, and then combining these five solutions. Once combined, the HMI mix was aliquoted into 50 ml tubes at a volume of 26.25 ml and placed into a -20°C freezer. The trypanosomes arrived frozen, and after thawing, were placed into a single, plastic 50 ml screw-capped tissue culture flask (BD Biosciences, San Jose, CA) containing 4 ml HMI-9 media and the flask was incubated for 24 hours in a 37°C 5% CO_2 incubator to establish culture. All trypanosome incubations were performed in this manner. Once a culture was established, 0.8 ml was placed into four separate 2 ml plastic external thread cryovials (Fisher Scientific, Pittsburg, PA) with 0.2 ml freezing medium and placed in a -80°C freezer for long term storage. Freezing medium consisted of 50% glycerol in cytomix (Morris, 2004). Cytomix was prepared by combining 120 mM KCl, (Sigma, St. Louis, MO) 0.15 mM $CaCl_2$ (Sigma, St. Louis, MO), 10 mM K_2HPO_4 (Sigma,

St. Louis, MO), 25 mM HEPES (Sigma, St. Louis, MO), 2 mM EDTA (Sigma, St. Louis, MO), 5 mM $MgCl_2$ (Sigma, St. Louis, MO) as per the recipe in Morris, 2004. The remaining 1 ml was subcultured into another 50 ml tissue culture flask containing 4 ml HMI media and incubated for 2 days. This process was repeated for the first three subcultures to ensure a large backup stock of early growth trypanosomes. After the first three subcultures, trypanosomes were subcultured every 2-3 days by taking 0.2 ml of the trypanosome culture and pipetting it into 4.8 ml of HMI-9 media to ensure the trypanosomes remained in the exponential growth phase and fresh growth media was readily available.

Antitrypanosomal Activity Assay Optimization

The activity of the plant extracts against the trypanosomes was determined using a PrestoBlue (Invitrogen, Carlsbad, CA) cell viability assay. All fluorescence readings were taken using a Spectramax M2 (Molecular Devices, Sunnyvale, California) fluorescent plate reader using an excitation wavelength of 560 nm, an emission wavelength of 590 nm, and a cutoff wavelength of 570 nm. The PrestoBlue assay was optimized for the trypanosomes and for laboratory conditions before any extracts were screened. Optimum incubation time of the trypanosomes in the 96-well plates was determined by seeding a clear 96-well plate (BD Biosciences, San Jose, CA) with 1×10^4 cells/well and measuring cell density using a hemocytometer every 8 hours for 96 hours. The optimum starting cell density and the maximum DMSO tolerance of trypanosomes was assessed by seeding plates with media only, 1×10^3 cells/well, 4×10^3 cells/well, and 1×10^4 cells/well and DMSO was at

concentrations of 10%, 1%, 0.5%, 0.1%, and 0%. The plates were incubated for 48 hours, and then 11 μl of PrestoBlue were added and allowed to incubate 24 h. The incubation time for trypanosomes with PrestoBlue was determined by seeding each well of a 96 well plate with 1 x 10^4 cells and allowing the plate to incubate for 48 h. After incubation, 11 μL of PrestoBlue was added and the plate was allowed to incubate as described above. Plates were checked visually every hour for 5 hours, and allowed to incubate for an additional 24 h before fluorescence readings were taken. Tests were also performed to ensure the trypanosome culture medium did not break down the PrestoBlue causing false readings. Ten wells were seeded with 100 μL of media and 10 wells with 1 x 10^4 cells. PrestoBlue (11μL) was added to each well and the plates were incubated for 48 h. The average relative fluorescence units from each set of wells were compared and examined for a 10-fold difference between the media wells and the cell wells. The positive control for antitrypanosomal activity screens was pentamidine isoethionate salt (Sigma, St. Louis, MO), a well known inhibitor of *Trypanosoma brucei* and one of the drugs currently used to treat African sleeping sickness. Wells containing pentamidine were included with every plate as a positive control.

Antitrypanosomal Activity Assay

For the initial activity assays, each plate was prepared by adding 90 μL of trypanosome culture at 10^5 cells/ml (~10^4 cells/well) and 10 μL of extract solution to the appropriate wells. Controls were included with each plate in triplicate and consisted of wells containing media only, untreated trypanosome culture,

trypanosome culture with 0.5% DMSO, and trypanosome culture with pentamidine. Extracts were prepared by adding 2 μL of extract solution to 38 μL of HMI-9 media. Once plates had been dosed they were incubated for 48 h. At the end of the incubation, 11 μL of PrestoBlue were added and the plates were allowed to incubate an additional 24 h before fluorescence readings were taken. Before each plate was transported to the fluorescence reader, 55 μL of 4% paraformaldehyde in PBS was added to each well containing trypanosomes. This ensured no live trypanosomes were ever outside the lab. The initial screening of extracts was done at a high dose of 50 μg/ml. Extracts that resulted an 85% reduction in fluorescence compared to the negative control were chosen as promising candidates and selected for IC_{50} determination.

L6 Rat Myoblast Cell Culture

L6 rat myoblast cells (ATCC# CRL-1458, Manassas, VA) were used to assess cell cytotoxicity and trypanosome selectivity of the plant extracts. The medium used to culture the L6 cells was prepared as recommended by the American Type Culture Collection by combining 450 ml of Dulbecco's Modified Eagle Media (DMEM) (Thermo Scientific, Waltham, MA), which contains 4.5 g/ml glucose, 4.00 mM L-glutamine, and sodium pyruvate, with 50 ml of heat inactivated fetal bovine serum (a 10% concentration), and then 50 units/ml of penicillin/streptomycin was added to the medium to help prevent bacterial contamination. The fetal bovine serum was heated in a water bath for 30 minutes at 56 °C to inactivate complement. For cultivation of L6 myoblast cells, 4.5 ml of cell culture medium were pipetted into

two separate 50 ml vented flasks and placed into a 37 °C CO_2 incubator for 15 min to allow the pH to adjust. After the 15 min incubation, the frozen L6 cells were thawed for 2 minutes in a 37°C water bath and 0.5 ml was added to each flask and placed into a 37 °C CO_2 incubator for 48 h. The L6 cells were maintained in 5 ml of the medium described above in 50 ml tissue culture flasks and incubated at 37 °C with 5% CO_2 as recommended by the ATCC. The rat myoblast cells were subcultured into new flasks every two or three days. To subculture the cells, all old medium was removed from the flask, and 2 ml of trypsin was added. After agitating for 2–3 seconds, the trypsin was quickly removed. This process inactivated any serum still in the flask. After the initial 2 ml of trypsin was removed, another 1 ml of trypsin was added and allowed to coat the bottom of the flask where the monolayer had formed. Once coated, the flasks were placed into the 37 °C 5% CO_2 incubator for 5 m, or until cells became free-floating and non-adherent. The flasks were then agitated to loosen any remaining adherent cells and remove them from the side of the flask. After the cells became suspended in the trypsin, 0.5 ml of the suspension was transferred to two new flasks containing 4.5 ml of media, and the remaining 0.5 ml was put into a waste container. If more than 2–3 days was needed between splitting cells, only 0.25 ml of cell solution was transferred to 4.75 ml of media and the remaining 0.75 ml was put into a waste container. The flask was then incubated as described previously. A seed lot was created from the first few passages by adding 0.5 ml of the trypsinized cells to two separate 50 ml flasks containing 4.5 ml of a DMEM and 5% (filter sterilized) DMSO solution. From these flasks, 1.0 ml was

pipetted into 10 cryovial tubes, 6 of which were stored in an -80 °C freezer, and 4 were stored in liquid nitrogen. This process was repeated 3 times resulting in 30 early seed lots. To revive frozen seed lots, the cryovials were allowed to thaw and 0.5 ml of cell culture was placed into 4.5 ml of new media. Media was replaced daily until the cells reached full confluency, at which point cells were cultured as described above. If cells were to be used to seed a 96-well plate, the same methods used to split cells were followed, except the 0.5 ml normally put into a waste container was placed into 1.5 ml of DMEM media. The concentration of this solution was calculated using a hemocytometer, and diluted to 5.5×10^4 cells/ml, or 5×10^3 cells/well.

L6 Rat Myoblast Cytotoxicity Activity Assay Optimization

The L6 rat myoblast cells were used to determine if plant extracts were cytotoxic to mammalian cells. All L6 cell assays were performed using black, clear-bottomed 96 well plates (Corning/Costar, Rochester, NY). Black plates were used to reduce possible background fluorescence from wells adjacent to the well being read. Before attempting the cytotoxicity experiments, tests were performed to assure optimal conditions for the experiments. The amount of incubation time the cells needed before cell metabolic activity plateaued was assessed by seeding five rows of wells with cells, and a fluorescence reading was taken at 0 h, 24 h, 48 h, 72 h, and 96 h. Plates were incubated for 72 h to ensure cells reached their maximum potential. Optimal incubation time for the PrestoBlue assay was also determined before any extract screens were performed. For this, a plate was seeded with 90 μL of cell

solution (5×10^3 cells/well) and allowed to incubate for 72 h. After incubation, 10 µL of PrestoBlue was added and a fluorescence reading was immediately taken. Additional readings were taken at 20 min, 45 min, 75 min, and 21 h. Tests were performed to ensure that the DMSO the extracts were suspended in, as well as the media, did not cause the PrestoBlue to break down, leading to inaccurate results. For this test, 90 µL of media and 10 µl of 10% DMSO were added to each well of a 96 well plate. The concentration used in the cytotoxicity experiments did not exceed 10%. As a control, one row of wells contained media only and no solvents. After all solvents had been added, PrestoBlue was added to all wells and plates were placed in a 37°C 5% CO_2 incubator for 30 min. Following incubation, fluorescent readings were taken on the plates. A control test was also performed to ensure the solvents did not inhibit cell growth. This test was performed in the same manner as the above solvent test, except each well was seeded with 5×10^3 L6 cells and allowed to incubate for 72 h before PrestoBlue was added. The chemical used as a positive control was podophyllotoxin (Sigma, St. Louis, MO) and a control test was included to show that the dose used in experimental conditions was enough to inhibit the cells. This test was performed by first seeding 12 wells with cells and allowing them to incubate overnight to adhere to the wells. After this incubation, a dose of 2.5 µg/well was added to 6 wells, at which point the plate was placed into a 37°C 5% CO_2 for 72 h.

At the end of the incubation period, PrestoBlue was added to each well and allowed to incubate for 30 min. At the end of the 30 min incubation, a fluorescence reading was taken on the plate.

L6 Mammalian Cell Cytotoxicity Assay

A high dose of 100 µg/ml was used to screen extracts for any activity against the L6 cells. Using a multipipetter, 90 µL of the cell solution (5000 cells/well) were added to each well of a 96 well plate and plates were incubated at 37°C 5% CO_2 for 3 h to allow the cells to attach to the surface of the well. At 3 h, extract dilutions were prepared by adding 4 µL of each extract to 36 µL of media to create a 1 µg/µl solution. Once extracts were prepared, 10 µL of the extract solution was added in triplicate to the designated wells. With each plate, 4 controls were also run in triplicate to ensure accurate results. The control wells contained media only (no cells), cells and media only, 10 µL of 10% DMSO, and the positive control wells contained 2.5 µg of podophyllotoxin. Once all extracts and controls were added, the plates were incubated for 72 h at 37°C 5% CO_2. At the end of the incubation, plates were examined under an inverted microscope to ensure normal control cell growth. If all controls were normal, 11 µL of PrestoBlue reagent was added to each well and allowed to incubate for 30 min. After the 30 min incubation a fluorescence reading was taken on the plates. Extracts were considered to be inactive against the L6 cells if the results showed less than 10% inhibition compared to negative controls.

Trypanosome IC_{50} of Suitable Plant Extracts

The extract concentration that inhibited trypanosome growth by 50% (IC_{50}) was determined for plant extracts that were active against trypanosomes and inactive against L6 cells. This assay was performed by seeding the appropriate wells of a 96 well plate with 1×10^4 trypanosomes and adding extracts in serial dilutions of 50, 25, 12.5, 6.25, 3.125, 1.56, 0.78, and 0.39 μg/ml. Once plates had been dosed, they were allowed to incubate for 48 h. At 48 h, 11 μl of PrestoBlue was added and plates were incubated an additional 24 h before reading. To determine the IC_{50}, the average fluorescence reading at each concentration was plotted as a graph and a line of best fit was calculated. The IC_{50} was estimated to be the X-value where Y=50.

Chapter III

Results

Results

Trypanosome Assay Incubation Time

The optimum incubation time of the trypanosomes for inhibition assays was determined to be 72 h (Figure 4). This incubation time allows for sufficient growth to produce measureable results, as well as provide the extracts adequate time to show activity. After ~80 hours, trypanosome populations diminish rapidly to a point where no results could be obtained.

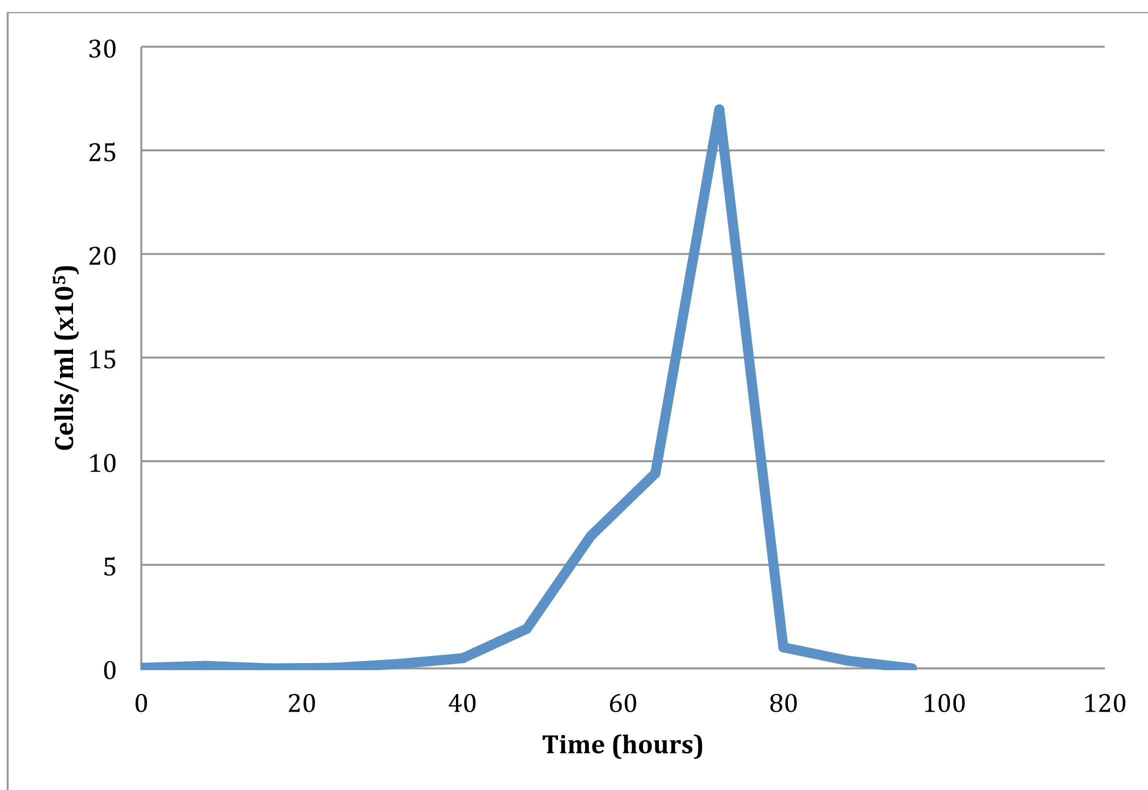


Figure 4. Cells/ml of trypanosomes over 96 h (starting cells/well was 4 x 10^3)

Trypanosome Assay Starting Cell Density and DMSO concentration

The DMSO-only control assay demonstrated the optimal cell density to be 1 x 10^4 cells/well and the maximum concentration of DMSO was 0.5%. All DMSO concentrations inhibited trypanosome growth at 1000 cells/well. A starting

concentration of 10% DMSO was inhibitory to all starting cell densities. DMSO concentrations at 1% inhibited trypanosome growth when starting with 1 x 10^4 cells, and always had a distinct inhibitory effect when starting with 4 x 10^3. The fluorescence readings obtained at 0.5% DMSO and below were comparable to negative controls (cells without DMSO) at a beginning density of 1 x 10^4 cells/well. Comparisons of cell densities at different levels of DMSO can be seen in Figure 5.

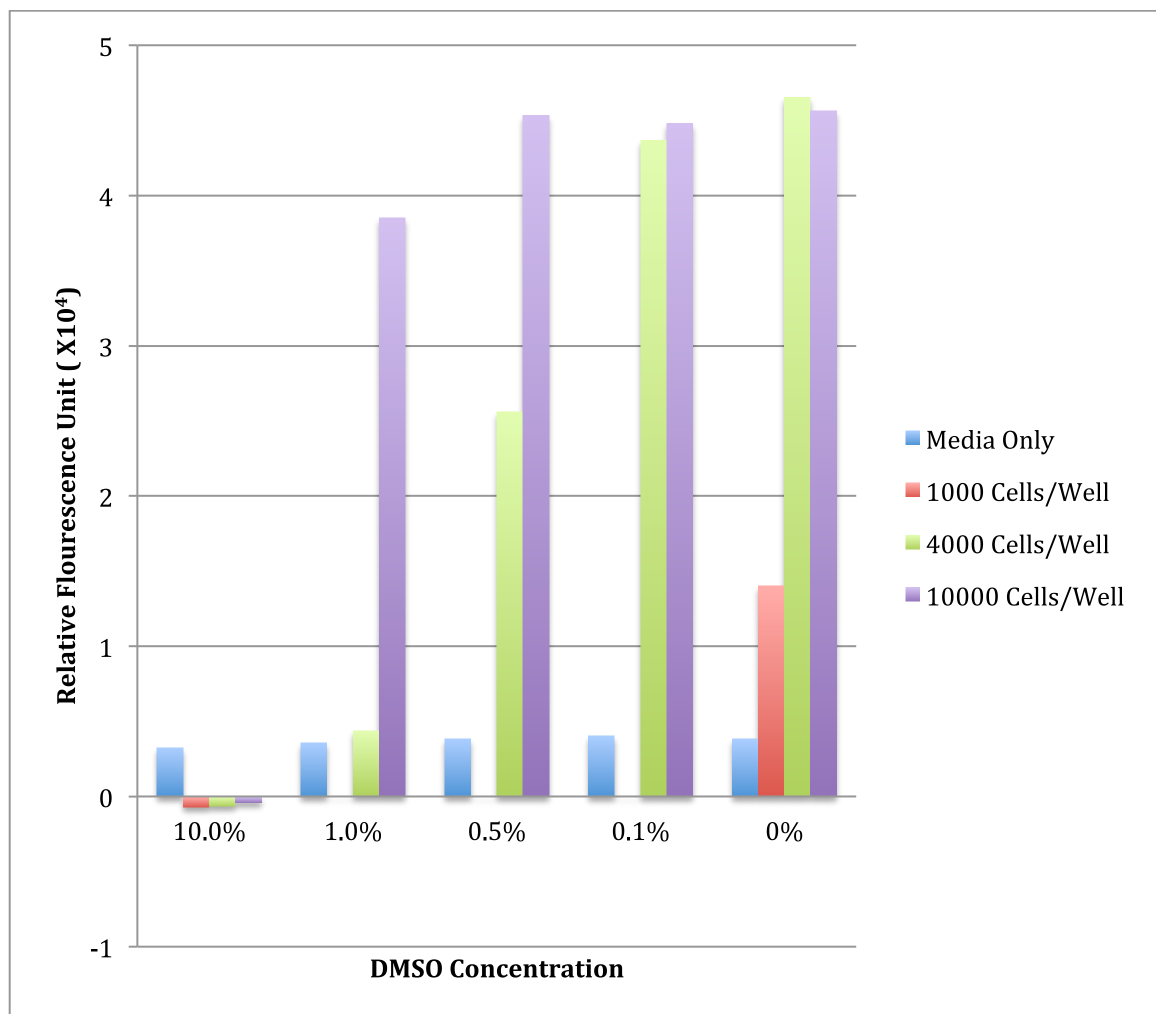


Figure 5. Relative fluorescence readings for different beginning cell densities and DMSO concentrations

Incubation time of Trypanosomes with PrestoBlue

For consistant reduction of resazurin in PrestoBlue by the strain *T. brucei brucei 427,* plates required 24 h incubation at 37 °C . When PrestoBlue had been reduced enough to obtain a fluorescence measurement, the color changed from blue to pink. When the plates were checked visually at points between 1–5 h, no visible change had taken place. At 24 h however, all test wells were reduced. To allow for a 24 h incubation with PrestoBlue and to end the assay at 72 h, PrestoBlue was added at 48 hours.

HMI-9 Media and Presto Blue

All trials of PrestoBlue and HMI-9 produced results of between 10–11 fold difference between the background produced by the media only and the reduction of PrestoBlue by media containing trypanosomes (Figure 6). To account for the small amount of background produced by the media, each plate had blank controls that were subtracted out before any calculations were performed on the data.

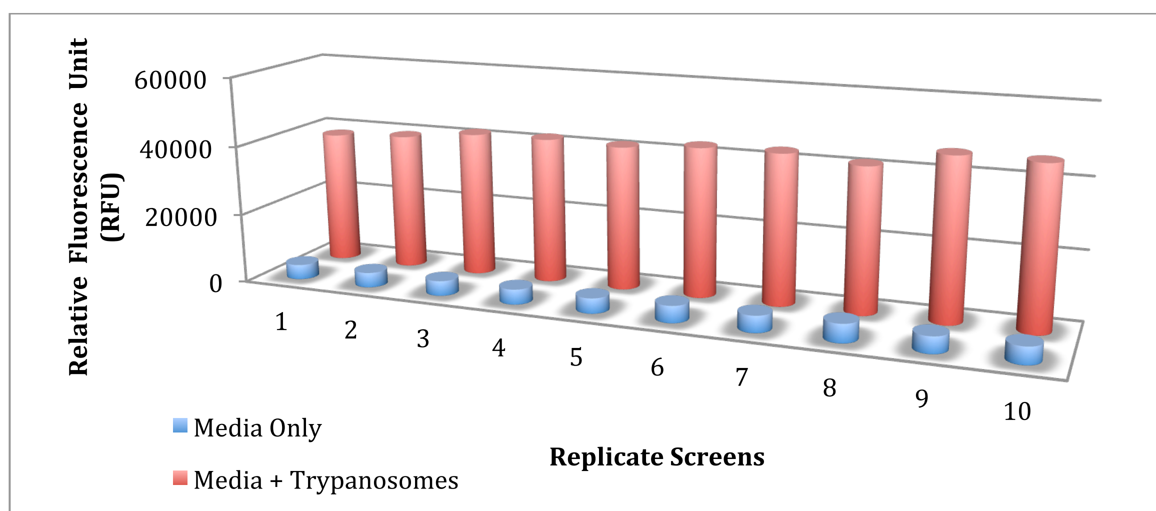


Figure 6. Comparison of RFUs from media and RFUs from live culture

L6 Cell Incubation Time

The L6 cells showed significant growth during the first 48 h, and only slight growth past this time point. The incubation time for the extract cytotoxicity assay was set at 72 h to ensure that the L6 cells reached their maximum density before the PrestoBlue was added. The flourescence readings of this assay can be seen in Figure 7.

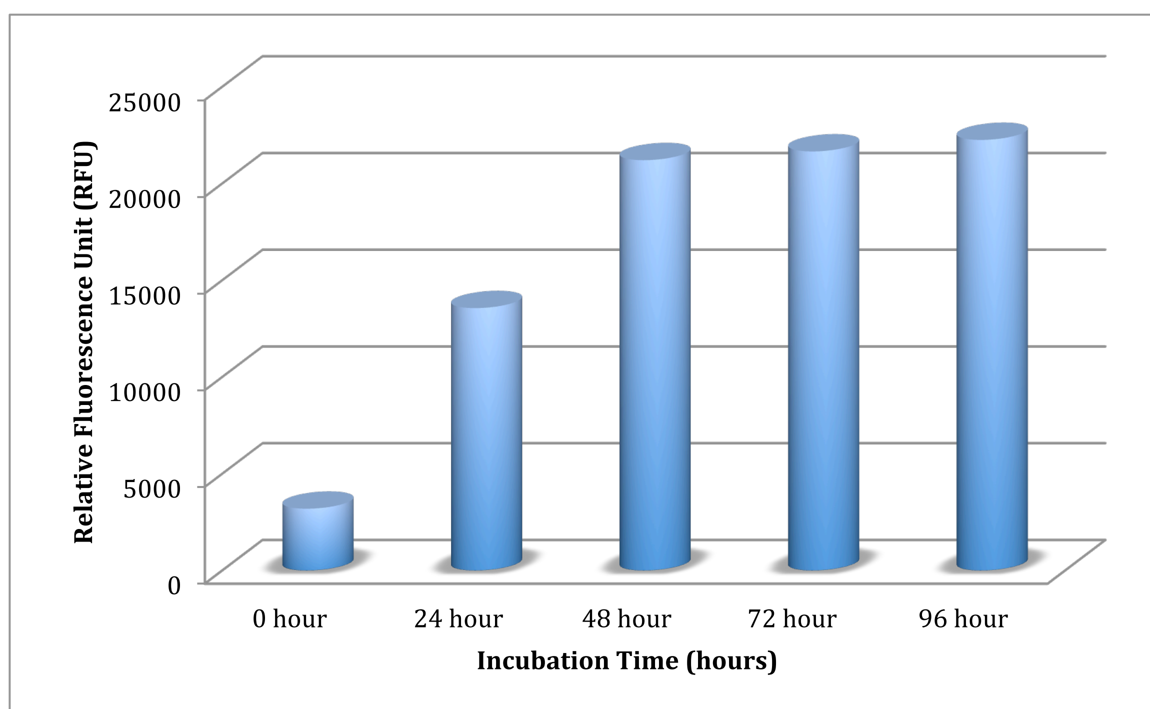


Figure 7. RFUs measured from L6 cells over 96 h

L6 Cell-PrestoBlue Incubation Time

PrestoBlue measurements plateaued at 20 min. For the cytotoxicity assay, a 30 min incubation with PrestoBlue was used. The results of the L6 cell incubation time assay are shown in Figure 8.

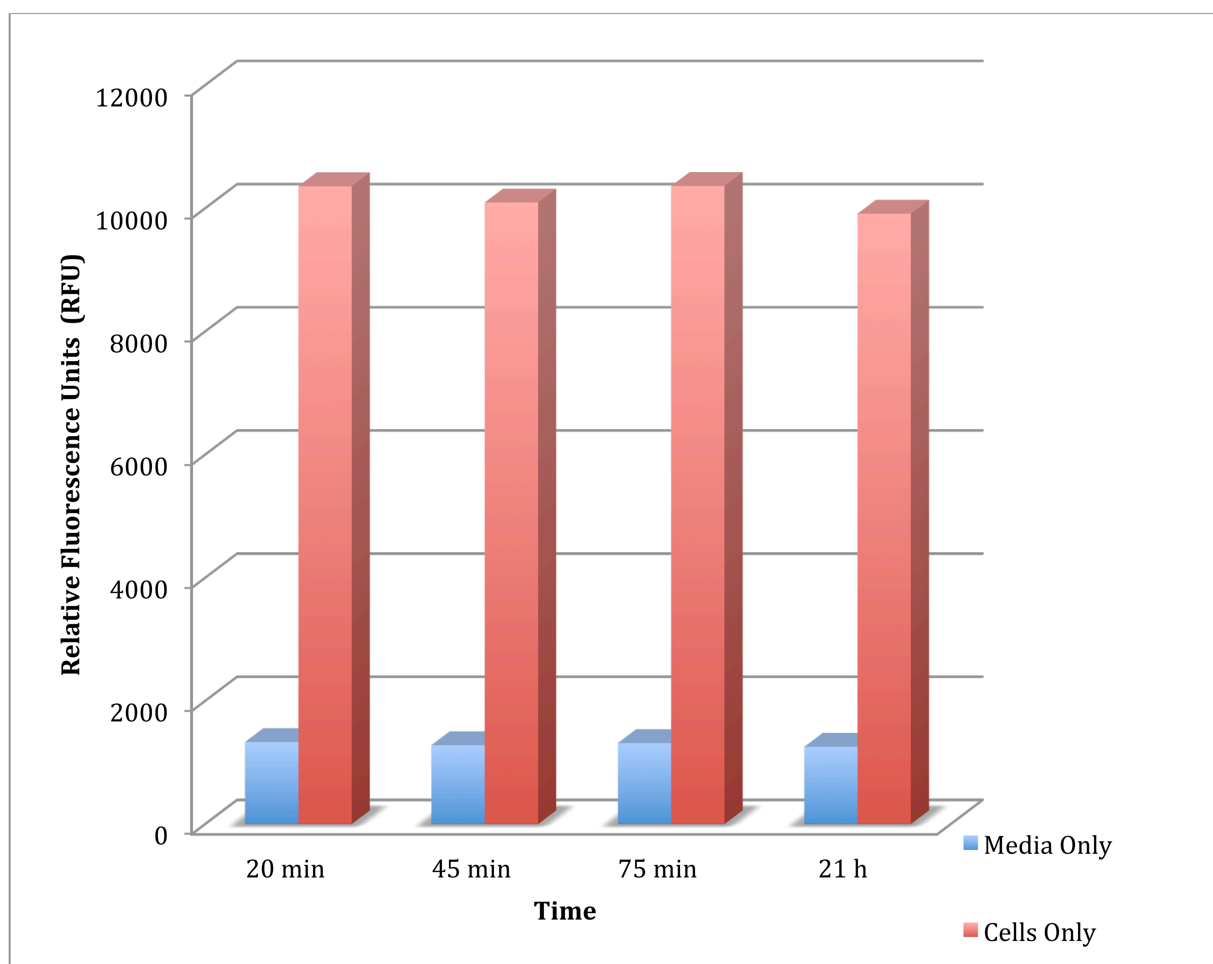


Figure 8. RFU from L6 cells and PrestoBlue across several time points

Effects of DMSO on L6 Cell Growth and PrestoBlue

The addition of 1% DMSO to the wells had no noticeable effect on the L6 cells' ability to grow, or to reduce PrestoBlue. The medium also had no reducing effects on PrestoBlue and the measurements obtained were between 30–40 fold less than wells containing cells. Any background produced by medium was measured with blank controls done in triplicate on each plate and the average was subtracted from each measurement before any calculations were performed. Effects of DMSO on cell growth and PrestoBlue reduction can be seen in Figure 9.

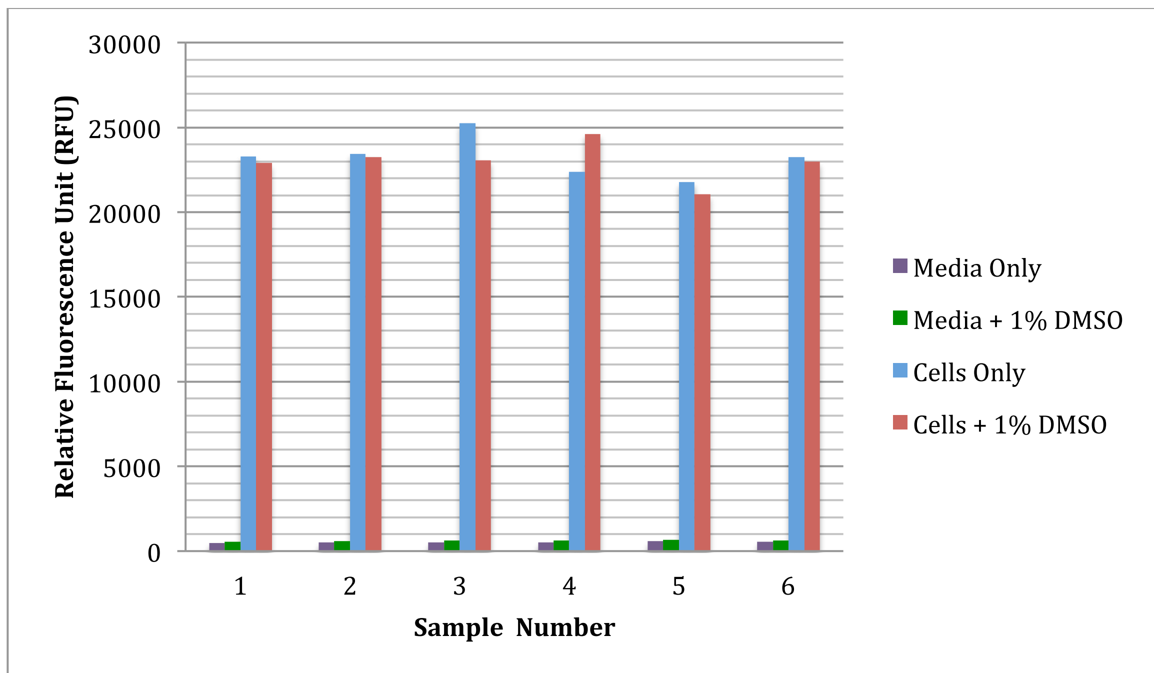


Figure 9. RFUs of media only, media + 1% DMSO, cells only, and cells + 1% DMSO

L6 Cell Inhibition by Podophyllotoxin

Podophyllotoxin reduced fluorescence measurements of L6 cells at concentrations as low as 2.5×10^{-4} µg/ml (Figure 10). The dose used in the assay was 10^4X greater than this. Control wells containing 2.5 µg podophyllotoxin were run in triplicate wells with each plate as a positive control.

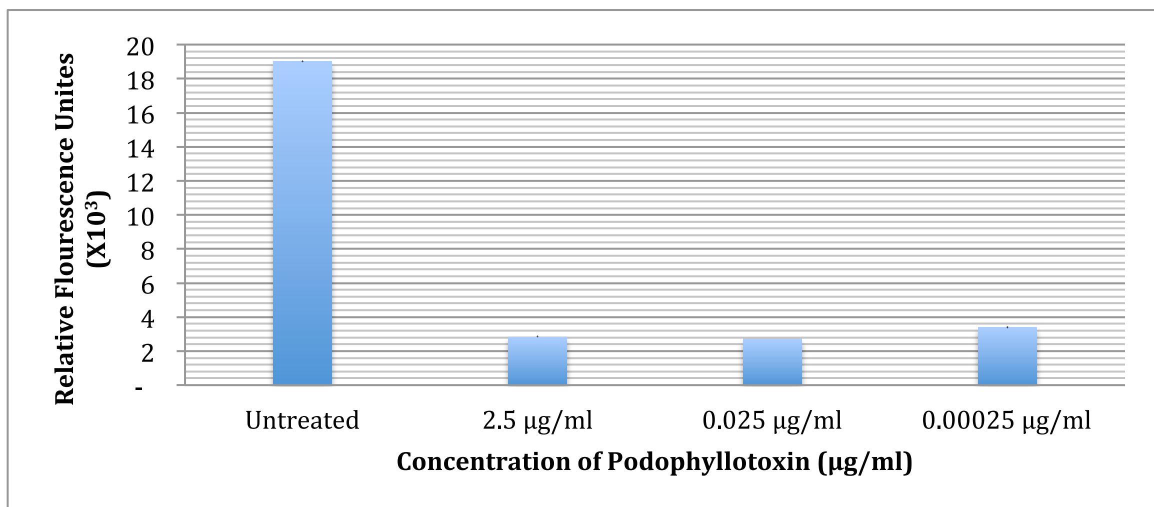


Figure 10. RFUs of untreated L6 cells compared to those with podophyllotoxin.

Initial Plant Extract Screening for Antitrypanosomal and Cytotoxic Activity

A total of 144 extracts were screened at 50 μg/ml against trypanosomes and at 100 μg/ml against L6 cells. Of the 144 extracts screened, 50 (34.7%) showed inhibition activity of greater than 85% against the trypanosomes, 52 extracts (36.1%) caused less than 10% inhibition against L6 cells, and 6 extracts (4.16%) fell within both categories (Figure 11). These were considered to be potential targets for further study. A full list of inhibition levels of all extracts against trypanosomes and L6 cells can be seen in Appendices II & III.

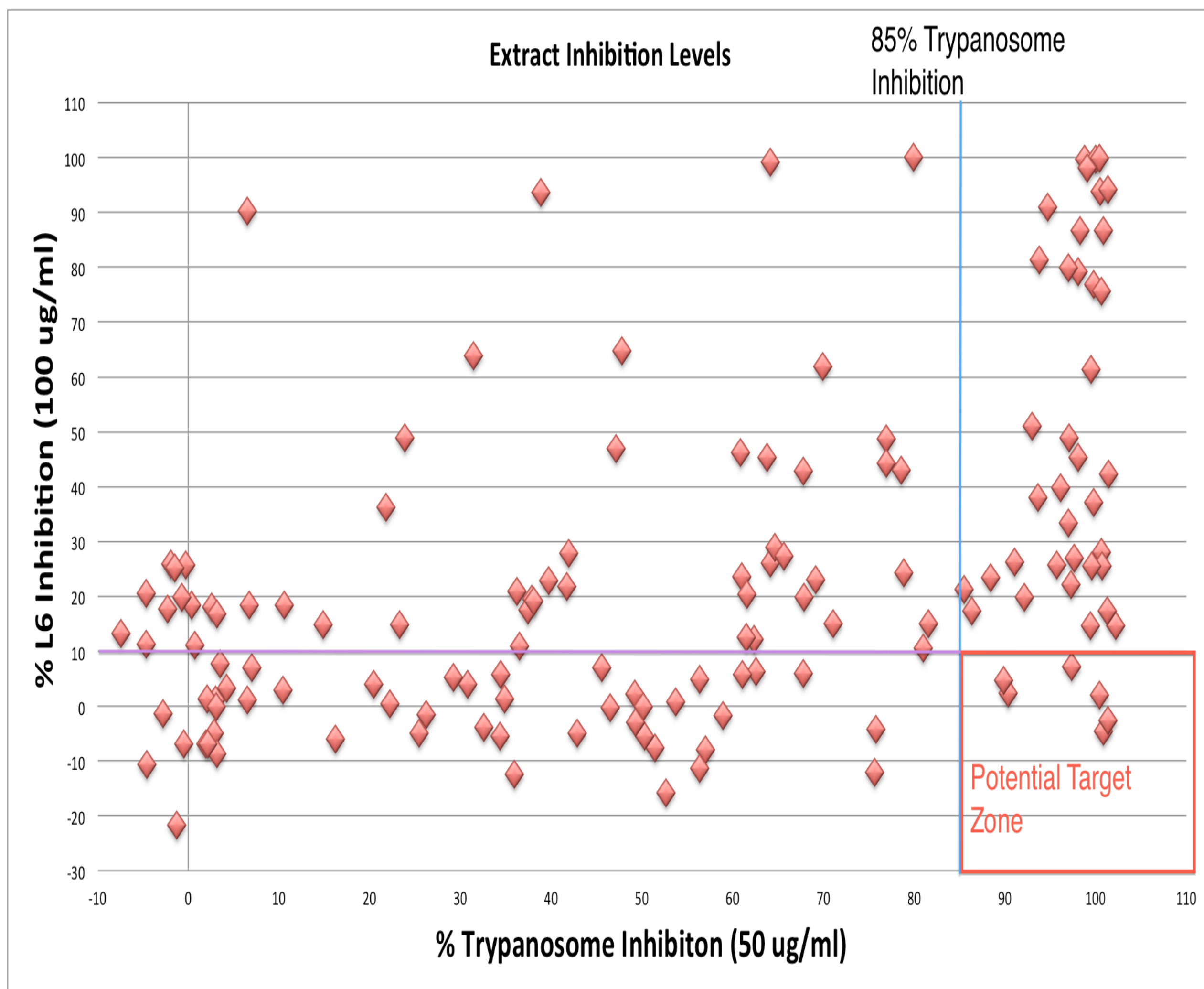


Figure 11. Extract activity against L6 cells and Trypanosomes

Potential Target IC_{50} of Trypanosomes

The petroleum ether extracts of *Elephantopus scaber* and *Psychotria rubra,* the ethanol extract of *Belamcanda chinensis*, the ethyl acetate extract of *Pandanus tectorius*, and the chloroform and water extracts of *Scutellaria baicalensis* all had their IC_{50} concentration determined by finding a line of best fit and solving for X at Y=50 (Figures 12–17). The extract IC_{50} values were calculated as follows: the petroleum ether extract of *Elephantopus scaber* had an IC_{50} of 46.65, the petroleum ether extract of *Psychotria rubra* had an IC_{50} of 38.02, the ethanol extract of *Belamcanda chinensis* had an IC_{50} of 38.51, the ethyl acetate extract of *Pandanus tectorius* had an IC_{50} of 37.73, the chloroform extract of *Scutellaria baicalensis* had an IC_{50} of 11.43, and water extract of *Scutellaria baicalensis* had an IC_{50} of 19.86.

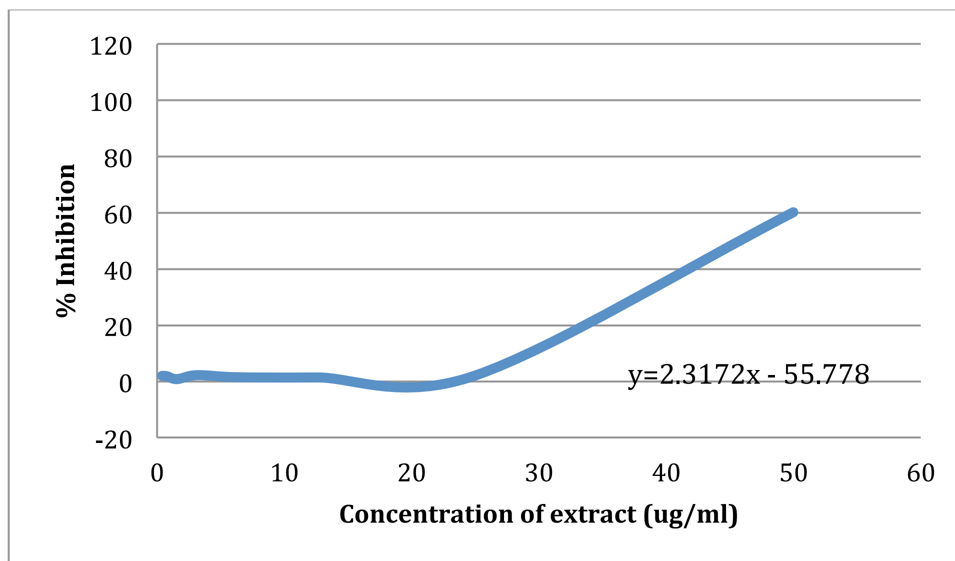


Figure 12. IC_{50} graph of the petroleum ether extract of *Elephantopus scaber*

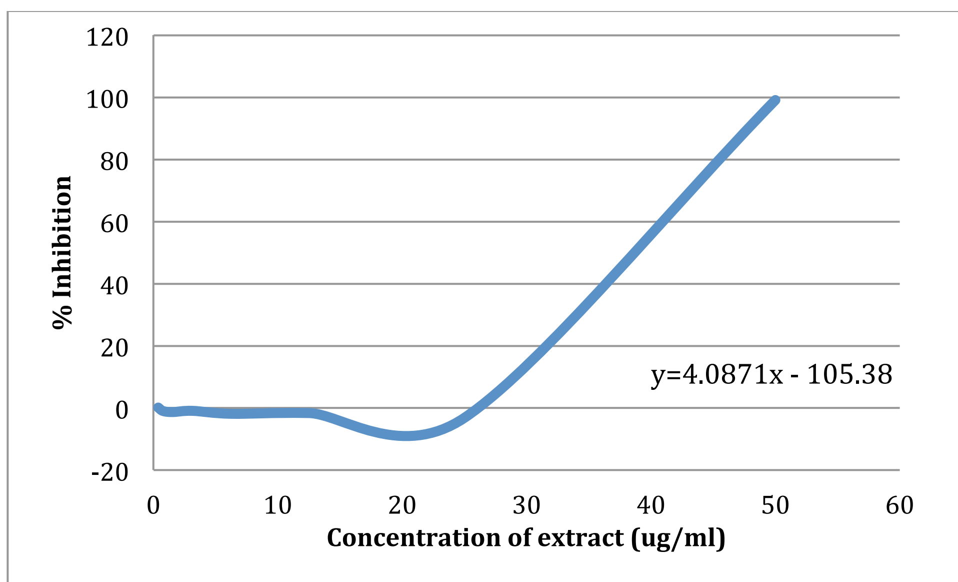


Figure 13. IC_{50} graph of the petroleum ether extract of *Psychotria rubra*

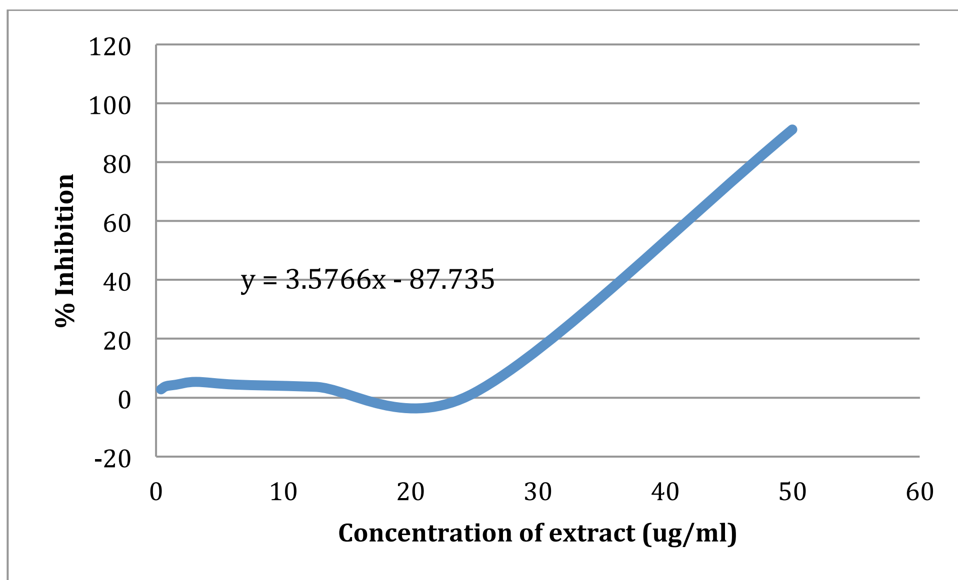


Figure 14. IC_{50} graph of the ethanol extract of *Belamcanda chinensis*

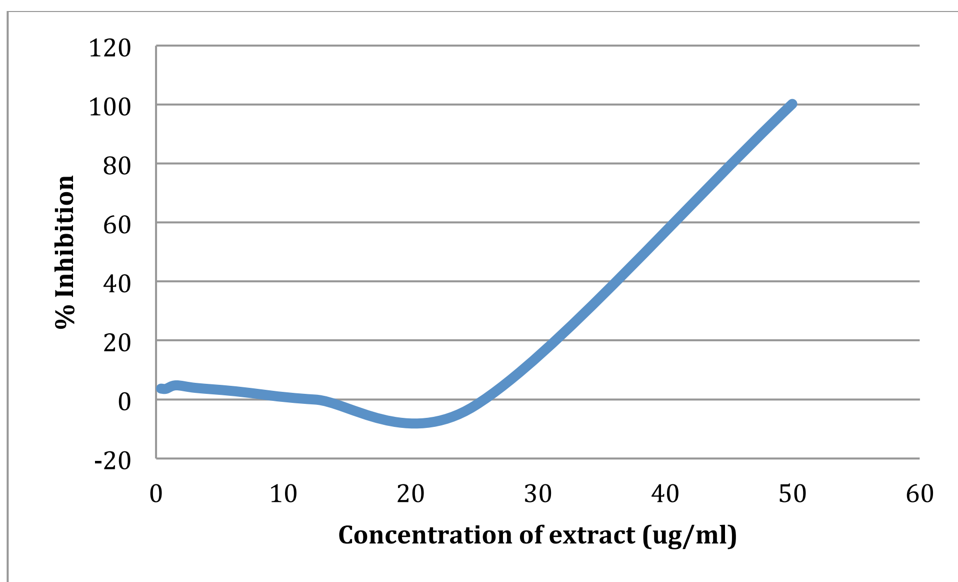


Figure 15. IC_{50} graph of the ethyl acetate extract of *Pandanus tectorius*

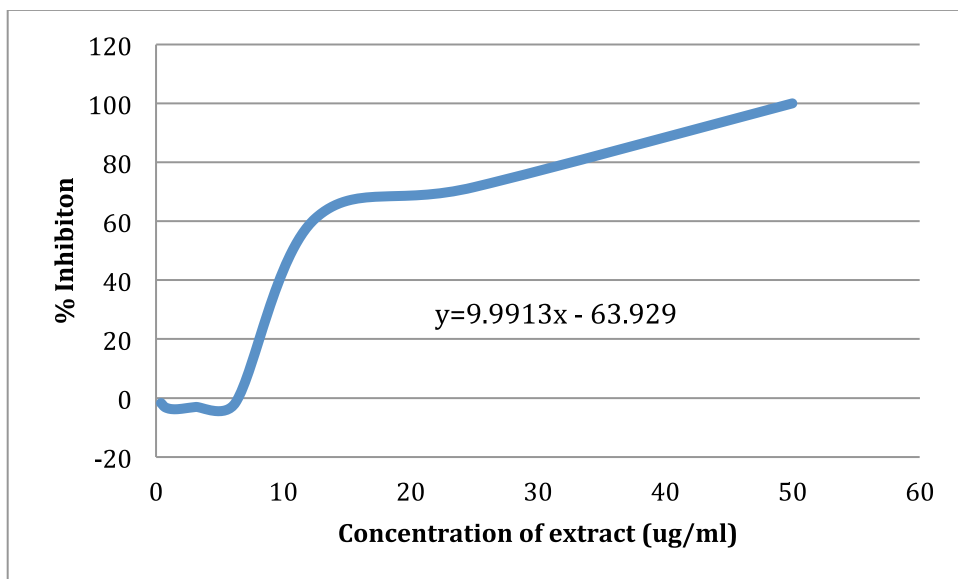


Figure 16. IC_{50} graph of the chloroform extract of *Scutellaria baicalensis*

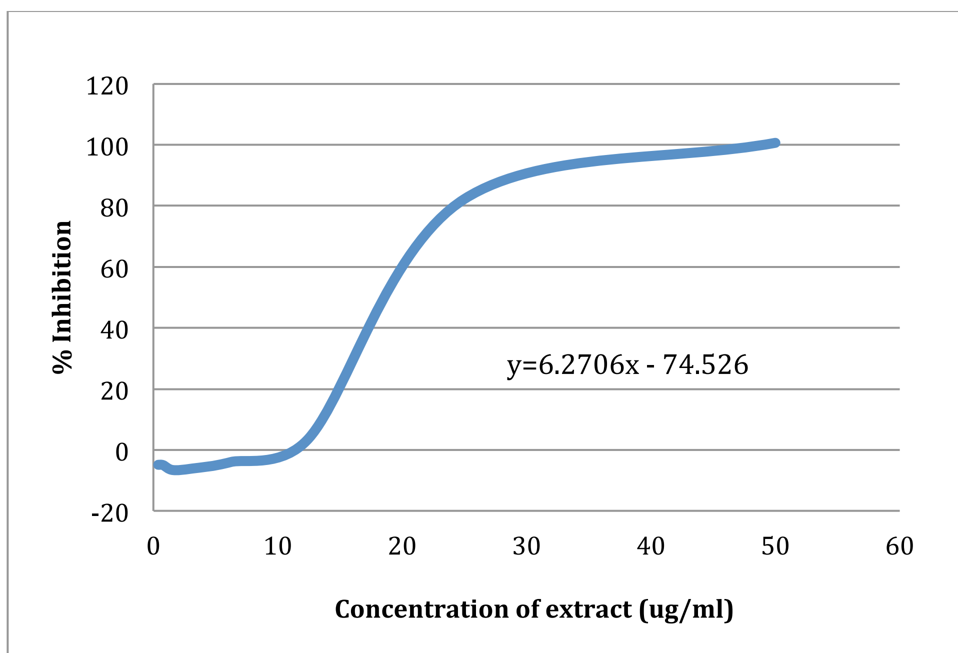


Figure 17. IC_{50} graph of the water extract of *Scutellaria baicalensis*

Chapter IV

Discussion

Discussion

African sleeping sickness continues to be a serious problem for those living in endemic areas. Its impact on the general health and economy of Africa shows a need for development of more effective treatments. The efficacy of current drugs to treat this disease is declining due to the emergence of resistant strains, and their toxicity makes them an undesirable solution (WHO, 2012). The currently used drugs can be difficult to obtain, and very little is known about their mode of action, despite their use for close to 100 years (Atouguia, 1999). Some of the treatments were not developed as a result of anti-trypanosomal research, but out of research into treatments for other diseases. The most recently developed drug, eflornithine, was originally produced to be an anti-tumor medicine, and is only effective against *T. brucei gambiense* (Atouguia, 1999). African trypanosomiasis generally occurs in areas where malnutrition and infection by other parasites, such as malaria, are common. The weakened physical state, combined with the severe side-effects of the anti-trypanosomal drugs, results in serious complications and morbidity (Atouguia, 1999).

In recent decades, development of new treatment for African trypanosomiasis has been lacking. Suramin, pentadimine, and melarsoprol were all developed in the first half of the 20th century (Atouguia, 1999). This lack of development calls for exploring new resources to identify potential drug candidates. Traditional Chinese medicine (TCM) has used herbal remedies to treat ailments for over 3000 years (Aiping, 2012). The Guangxi Botanical Garden of Medicinal Plants

has over 2,100 different species of plants used in TCM. Newer technologies such as high-throughput screening (HTS) have promoted drug discovery by allowing testing of many different compounds in a short amount of time (Chapman, 2004). A format commonly used for HTS assays is the 96-well microtiter plate to screen multiple compounds at once, which is the method implemented in this study. It was observed that if trypanosomes were grown in black walled microtiter plates, the results were highly variable. Even when no extracts or other compounds were added to the wells, some wells showed hindered growth, and others showed no growth at all. Switching to clear-walled microtiter plates solved this problem. This suggests that a compound found in the black-walled plates has an adverse effect on the trypanosomes. The black-walled plates had no observable effect on the L6 rat skeletal myoblast cell line, indicating the plates were affecting something specific to the trypanosomes.

Using HTS and 96-well plates, 144 extracts from plants used in TCM were screened against *Trypanosoma brucei* and the rat skeletal myoblast L6 cell line. There were clearly differential effects of the plant extracts on the trypanosomes, as well as the myoblasts. The results showed that six extracts effectively inhibited trypanosome growth with minimal cytotoxic effects on the L6 cells. The high level of inhibition of these extracts compared to the negative control indicates that compounds with potential therapeutic value for the treatment of *T. brucei* infection could be present within these extracts.

The most likely candidates are extracts that displayed particularly high inhibitory properties against the trypanosomes (greater than 85%), while having

minimal cytotoxic effects on the L6 cells (less than 10%). Six extracts were identified from 5 different plants (*Elephantopus scaber, Psychotria rubra, Belamcanda chinensis, Pandanus tectorius, Scutellaria baicalensi.*). All of these extracts exhibited activity selective for trypanosomes when compared to the mammalian cells. An IC_{50} was not calculated for the L6 cells in this study. Acquiring an IC_{50} for the L6 myoblast cells would require DMSO concentrations greater than 1% that would, in itself, be inhibitory. Because of this, extracts were unable to be prepared at high enough concentrations to obtain a true IC_{50}. As a result, all L6 cell IC_{50} numbers are reported as > 100 µg/ml.

The extract that showed the highest amount of activity against *T. brucei* was from *Scutellaria baicalensis*. This plant has been used in the treatment of cancer, as well as pathogenic infections of the respiratory and gastrointestinal tracts (Jung, 2012). It is the only plant that had two biologically active extracts, and these extracts displayed the two lowest IC_{50} values. The *Scutellaria baicalensis* chloroform extract displayed the lowest IC_{50} at a concentration of 11.43 µg/ml. Chloroform is a non-polar solvent, so the compounds within this extract are more than likely non-polar themselves (Umar, 2013). Chloroform has recently been shown to extract compounds such as alkaloids, tannins, saponins, and glycosides from plant matter, so these types of compounds may be present within this extract (Biswas, 2012). The water extract also had a high activity level against trypanosomes with an IC_{50} of 19.56 µg/ml. As water was used as the solvent, and water is a polar molecule, the components of this extract are probably polar compounds (Umar, 2013). When

water is used as a solvent to prepare plant extracts, it has been observed to yield resins, flavonosides, and saponins, and these types of compounds from other plants have been shown to have antitrypanosomal effects (Atawadi, 2007). The IC_{50} graphs for this plant can be seen in Figure 16 and Figure 17. *Scutellaria baicalensis* had active components in both polar and non-polar solvents. This suggests that this plant has a range of compounds with antitrypanosomal activity. Pertaining to the L6 myoblast cell line, these two extracts were the only ones out of the 6 potential candidates to cause RFU values greater than the negative control, suggesting they may possibly have a mitogenic effect on the mammalian cell line.

The third highest level of activity came from the petroleum ether extract obtained from the branches and leaves of the plant *Psychotria rubra.* This plant has been shown to have anti-microbial activity against bacteria such as *Staphylococcus aureus, Psuedomonas aeruginosa,* and *Shigella sonnei,* and has been indicated to contain antitumor compounds (Giang, 2007, Hayashi, 1987). The active extract from this plant displayed an IC_{50} of 38.02 μg/ml against trypanosomes (Figure 13) and was obtained using the non-polar solvent petroleum ether, indicating the compounds within this extract are probably non-polar as well (Umar, 2013). Petroleum ether has also been shown to yield sterols and triterpenes, suggesting these might be possible components of the extract (Atawadi, 2007).

The extracts created from the plant *Pandanus tectorius* were obtained from the stem of the plant only. This is another plant used in TCM that has been the subject of scientific research, particularly for its inhibitory properties against

Mycobacterium tuberculosis (Tan, 2008). Extract based assays have shown this plant to have antimicrobial phytosterols active against *Mycobacterium tuberculosis (Tan, 2008)*. The desired activity in this assay was seen in the extract that was prepared using ethyl acetate as the solvent. Ethyl acetate is a polar solvent, so the active molecules within this extract are probably polar compounds. Ethyl acetate has also been shown to extract phenolic compounds as well (Lim, 2005). The extract had an IC_{50} of 37.73 μg/ml (Figure 15).

Elephantopus scaber is a member of the Asteraceae family and has been shown to have anti-tumor, anti-viral, and anti-inflammatory effects (Geng, 2011, Geetha, 2010). The extract from *Elephantopus scaber* that displayed desirable biological activity was the petroleum ether extract, and had an IC_{50} of 45.65 μg/ml (Figure 12). Because petroleum ether was used for this extract, the compounds within the extract are more than likely non-polar molecules, (Umar, 2013).

The sixth extract of interest came from the plant *Belamcanda chinensis*, a plant that has been readily used in TCM to treat pharyngitis, asthma, and cancer (Lui, 2012). This extract had an IC_{50} of 38.51 μg/ml and was prepared with 95% ethanol (Figure 14). Ethanol has both polar and non-polar properties, so the components of this extract probably consist of both polar and non-polar compounds. In previous studies, ethanol has also been shown to yield tannins and alkaloids with antitrypanosomal properties from plant extractions (Mbaya, 2011).

The plants that showed marked (> 85%) biological activity against *T. brucei* have been shown to have compounds of potential medicinal use for treating viral

infections and cancer. This indicates that the library of plants used in TCM may have potential in the development of new drugs for treatment of *T. brucei*. Antitrypanosomal assays using the plants identified above have not been described in the literature before. My results suggest that future studies should be conducted to identify the specific active compound found within each extract. This study was performed using crude extracts that contain numerous compounds within each extract. While the IC_{50} of the extract was seen as micrograms/ml, the concentration of the actual active component could potentially be much less. The mode of action by which the extracts enact their biological activity is beyond the scope of this investigation. Once specific active compounds are identified, the mode of action should be determined. These extracts were screened against only a single mammalian cell line, and research could be conducted to ensure that the candidate extracts and their components are non-toxic to other mammalian cell lines and pose a low risk for complications. Differences in *T. brucei* strains may cause discrepancies in the effectiveness of compounds to eliminate them. This has already been seen with the drug eflornithine, a treatment that is only effective against the gambiense strain. In the current study the assay was performed using *Trypanosoma brucei brucei* strain 427. Further research should be performed testing these extracts, or the isolated compounds found within them, against other strains of *Trypanosoma brucei* to determine if any specificity of activity exists.

In summary, searching plants used in traditional medicine for unique compounds capable of inhibiting trypanosomes could lead to safer and more effective treatments of *T. brucei*. Results from the current study support this concept.

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Appendices

Appendix I

Plant Extract List

Plant	Part of plant	Code on tube	Solvent	Note
Berchemia lineate (Linn.) DC. 铁包金	root	BELI-W BELI-PE BELI-E BELI-EA	Water Ether Ethanal Ethyl acetate	antitumor, anti-inflammatory and analgesic
Antirrhinum majus Linn. 金鱼草	the whole plant	ANMA-W ANMA-PE ANMA-E ANMA-EA	Water Ether Ethanal Ethyl acetate	anti-inflammatory, anti-tumor, anti-ulcer
Consolida ajacis L. 飞燕草	the whole plant	DEGR-W DEGR-PE DEGR-E DEGR-EA	Water Ether Ethanal Ethyl acetate	Tooth pain, pestcide, antibacterial, antifungus
Euphorbia lathyris Linn. 续随子	the whole plant	EULA-W EULA-PE EULA-E EULA-EA	Water Ether Ethanal Ethyl acetate	Diuretic,anti-parasite(schistosomes), anti- cancer, purgative, rubefacient, TOXIC
Fluggea virosa (Willd.) Baill. 白饭树	stem	FLVI-W FLVI-PE FLVI-E FLVI-EA	Water Ether Ethanal Ethyl acetate	Snakebites, contraceptive, STDs, rashes, anti-malarial, diarrhea, pneumonia
Papaver rhoeas L. 虞美人	the whole plant	PARH-W PARH-PE PARH-E PARH-EA	Water Ether Ethanal Ethyl acetate	Diarrhea
Mallotus repandus (Willd.) Muell. Arg. 石岩枫	the whole plant	MARE-W MARE-PE MARE-E MARE-EA	Water Ether Ethanal Ethyl acetate	anti-inflammatory, pain
Ricinus microcarpus G. M. Popova. 意大利蓖麻	the whole plant	RICO-W RICO-PE RICO-E RICO-EA	Water Ether Ethanal Ethyl acetate	"Caster Oil Plant". Laxative, burns, acne
Averrhoa caram bola L. (Carambola) 杨桃根	root	TEA144 TBU72 TBU846	Chloroform Methanol Acetone	diabetes
Millettia Pulchra		MP	Acetone	cardiovascular disease
Costustoot 木香		Cla	Chloroform	stomach disease
Scutellaria baicalensis		Sb-1	Ethanol	anti-microbial, anti-

Plant	Part of plant	Code on tube	Solvent	Note
Georgi 黄芩		Sb-2	Petroleum ether	tumor, anti-inflammation
		Sb-3	Chloroform	
		Sb-4	Ethyl acetate	
		Sb-5	Butanol	
		Sb-6	Water	
Cyclocarya paliurus 青钱柳		cp-1	Ethanol	anti-bacteria, anti-tumor, anti-inflammation, anti-oxidation, reduce blood sugar (hypoglycemic activity), reduce cholesterol, lower blood pressure,
		cp-2	Petroleum ether	
		cp-3	Chloroform	
		cp-4	Ethyl acetate	
		cp-5	Butanol	
		cp-6	Water	
Lithocarpus polystachyus Rehd 多穗柯		lp-1	Ethanol	reduce blood sugar, reduce cholesterol, lower blood pressure,, anti-bacteria
		lp-2	Petroleum ether	
		lp-3	Chloroform	
		lp-4	Ethyl acetate	
		lp-5	Butanol	
		lp-6	Water	
Catsia tora Linn 决明子		ct-1	Ethanol	reduce cholesterol, lower blood pressure
		ct-2	Petroleum ether	
		ct-3	Chloroform	
		ct-4	Ethyl acetate	
		ct-5	Butanol	
		ct-6	Water	
Paeonia suffruticosa	seeds	mdz-1	92% Ethanol	Anti-tumor, anti-oxidation, anti-inflammation
		mdz-2	70% Ethanol	
		mdz-3	Petroleum ether	
		mdz-4	Chloroform	
		mdz-5	Ethyl acetate	
		mdz-6	Water	
Garcinia paucinervis Chun et How	stem, branch, leaf	1A	petroleum ether	anti-microbial, anti-tumor, anti-inflammation
		1B	ethyl acetate	
		1C	95% ethanol	
		1D	water	

Plant	Part of plant	Code on tube	Solvent	Note
Elephantopus scaber Linn.	branch, leaf	2A	petroleum ether	anti-microbial, anti-tumor, anti-inflammation
		2B	ethyl acetate	
		2C	95% ethanol	
		2D	water	
Psychotria rubra (Lour.) Poir.	branch, leaf	3A	petroleum ether	anti-microbial, anti-tumor, anti-inflammation
		3B	ethyl acetate	
		3C	95% ethanol	
		3D	water	
Evodia lepta (Spreng.) Merr.	branch, leaf	4A	petroleum ether	anti-microbial, anti-tumor, anti-inflammation
		4B	ethyl acetate	
		4C	95% ethanol	
		4D	water	
Mussaenda pubescens Ait. f	branch, leaf	5A	petroleum ether	anti-microbial, anti-tumor, anti-inflammation
		5B	ethyl acetate	
		5C	95% ethanol	
		5D	water	
Glycosmis citrifolia (Willd.) Lindl.	stem	6A	petroleum ether	anti-microbial, anti-tumor, anti-inflammation
		6B	ethyl acetate	
		6C	95% ethanol	
		6D	water	
Achras zapota Linn.	branch, leaf	7A	petroleum ether	anti-microbial, anti-tumor, anti-inflammation
		7B	ethyl acetate	
		7C	95% ethanol	
		7D	water	
Polygonum perfoliatum L.	branch, leaf	8A	petroleum ether	anti-microbial, anti-tumor, anti-inflammation
		8B	ethyl acetate	
		8C	95% ethanol	
		8D	water	
Stephania longa Lour.	the whole plant	9A	petroleum ether	anti-microbial, anti-tumor, anti-inflammation
		9B	ethyl acetate	
		9C	95% ethanol	
		9D	water	

Plant	Part of plant	Code on tube	Solvent	Note
Belamcanda chinensis (Linnaeus) Redoute	the whole plant	10A	petroleum ether	anti-microbial, anti-tumor, anti-inflammation
		10B	ethyl acetate	
		10C	95% ethanol	
		10D	water	
Crocosmia crocosmiflora (Nichols.) N. E. Br.	the whole plant	11A	petroleum ether	anti-microbial, anti-tumor, anti-inflammation
		11B	ethyl acetate	
		11C	95% ethanol	
		11D	water	
Pandanus tectorius Sol.	stem	12A	petroleum ether	anti-microbial, anti-tumor, anti-inflammation
		12B	ethyl acetate	
		12C	95% ethanol	
		12D	water	
Eupatorium odoratum L.	aerial parts	13A	petroleum ether	anti-microbial, anti-tumor, anti-inflammation
		13B	ethyl acetate	
		13C	95% ethanol	
		13D	water	
Euphorbia hirta Linn.	the whole plant	14A	petroleum ether	anti-microbial, anti-tumor, anti-inflammation
		14B	ethyl acetate	
		14C	95% ethanol	
		14D	water	
Aristolochia tagala Cham.	the whole plant	15A	petroleum ether	anti-microbial, anti-tumor, anti-inflammation
		15B	ethyl acetate	
		15C	95% ethanol	
		15D	water	
Bidens biternata (Lour.) Merr. et Schreff	aerial parts	16A	petroleum ether	anti-microbial, anti-tumor, anti-inflammation
		16B	ethyl acetate	
		16C	95% ethanol	
		16D	water	
Cyperus rotundus L.	the whole plant	17A	petroleum ether	anti-microbial, anti-tumor, anti-inflammation
		17B	ethyl acetate	
		17C	95% ethanol	
		17D	water	

Plant	Part of plant	Code on tube	Solvent	Note
Gnetum parvifolium (Warb.) C. Y. Cheng	vine, leaf	18A	petroleum ether	anti-microbial, anti-tumor, anti-inflammation
		18B	ethyl acetate	
		18C	95% ethanol	
		18D	water	
Antirrhinum majus Linn.	the whole plant	19A	petroleum ether	anti-microbial, anti-tumor, anti-inflammation
		19B	ethyl acetate	
		19C	95% ethanol	
		19D	water	
Catharanthus roseus (Linn.) G. Don	the whole plant	20A	petroleum ether	anti-microbial, anti-tumor, anti-inflammation
		20B	ethyl acetate	
		20C	95% ethanol	
		20D	water	
Microsorium fortuni (Moore) Ching	aerial parts	21A	petroleum ether	anti-microbial, anti-tumor, anti-inflammation
		21B	ethyl acetate	
		21C	95% ethanol	
		21D	water	

Appendix II

High Throughput Screen of Plant Extracts Against *T. brucei*

Extract ID	RFU 1	RFU 2	RFU 3	Sample Mean	stdev	SEM	% inhitbition
Media Only	4771	4748	4705	4,741	34	19	N/A
Untreated	32911	36836	33782	34,510	2,061	1190	-
.5% DMSO	34301	36969	38341	36,537	2,054	1186	-5.87
Pentamidine	-137	-154	-221	(171)	44	26	100.49
1A	340	847	763	650	272	157	98.12
1B	6715	11825	18579	12,373	5,951	3436	64.15
1C	18515	32375	33083	27,991	8,214	4742	18.89
1D	8053	15302	17113	13,489	4,794	2768	60.91
2A	1604	816	270	897	671	387	97.40
2B	-406	-722	-660	(596)	167	97	101.73
2C	21414	21152	36784	26,450	8,950	5168	23.35
2D	444	193	625	421	217	125	98.78
3A	-81	-229	-147	(152)	74	43	100.44
3B	11240	11799	13569	12,203	1,216	702	64.64
3C	2643	2380	2207	2,410	220	127	93.02
3D	-64	885	1621	814	845	488	97.64
4A	139	56	25	73	59	34	99.79
4B	2749	-51	75	924	1,581	913	97.32
4C	11411	13365	14155	12,977	1,413	816	62.40
4D	6327	12575	13034	10,645	3,747	2163	69.15
5A	34670	31529	20474	28,891	7,457	4305	16.28
5B	35586	33690	33863	34,380	1,048	605	0.38
5C	35320	36379	34153	35,284	1,113	643	-2.24
5D	3665	38	672	1,458	1,937	1118	95.77
6A	16246	18615	15367	16,743	1,680	970	51.48
6B	7491	9189	7212	7,964	1,070	618	76.92
6C	20893	20948	18478	20,106	1,410	814	41.74
6D	33220	33583	21316	29,373	6,980	4030	14.88
7A	16591	17599	17272	17,154	514	297	50.29
7B	13035	8694	9375	10,368	2,335	1348	69.96
7C	36552	35304	36443	36,100	691	399	-4.61
7D	15437	14275	10632	13,448	2,507	1447	61.03

Extract ID	RFU 1	RFU 2	RFU 3	Sample Mean	stdev	SEM	% inhitbition
Media Only	4740	4759	4717	4,739	21	12	NA
Untreated	36889	40044	38668	38,534	1,582	913	-
.5% DMSO	38748	37986	39133	38,622	584	337	-0.23
Pentamidine	-183	-130	-198	(170)	36	21	100.44

Extract ID	RFU 1	RFU 2	RFU 3	Sample Mean	stdev	SEM	% inhitbition
8B	14081	10519	12534	12,378	1,786	1031	67.88
8C	13690	12229	13752	13,224	862	498	65.68
8D	7669	7396	9639	8,235	1,224	707	78.63
9A	8273	11477	10739	10,163	1,678	969	73.63
9B	-327	-364	-358	(350)	20	11	100.91
9C	-491	-570	-593	(551)	54	31	101.43
9D	19610	21271	34417	25,099	8,112	4683	34.86
10A	-262	-274	-258	(265)	8	5	100.69
10B	-72	39	10	(8)	58	33	100.02
10C	4058	3516	3559	3,711	301	174	90.37
10D	20185	33612	36616	30,138	8,749	5051	21.79
11A	18181	15147	16346	16,558	1,528	882	57.03
11B	8674	11886	16672	12,411	4,025	2324	67.79
11C	-249	-109	-125	(161)	77	44	100.42
11D	3804	20727	36521	20,351	16,362	9446	47.19
12A	33775	20992	19306	24,691	7,912	4568	35.92
12B	326	1690	3428	1,815	1,555	898	95.29
12C	19991	18314	20341	19,549	1,083	626	49.27
12D	14939	19944	32233	22,372	8,899	5138	41.94
13A	5327	3889	4153	4,456	765	442	88.44
13B	1224	553	5365	2,381	2,606	1505	93.82
13C	448	3533	20430	8,137	10,757	6211	78.88
13D	1076	1086	520	894	324	187	97.68
14A	20928	20412	19533	20,291	705	407	47.34
14B	-382	-152	-247	(260)	116	67	100.68
14C	215	514	16007	5,579	9,032	5215	85.52
14D	-1072	686	3793	1,136	2,463	1422	97.05
15A	967	17279	36493	18,246	17,783	1026 7	52.65

Extract ID	RFU 1	RFU 2	RFU 3	Sample Mean	stdev	SEM	% inhitbition
Media Only	4750	4178	4608	4,512	298	172	NA
Untreated	36213	39386	38291	37,963	1,612	931	-
.5% DMSO	20798	38061	38565	32,475	10,115	5840	14.46
Pentamidine	382	127	215	241	130	75	99.36
15B	339	-167	24	65	256	148	99.83
15C	16787	19926	31925	22,879	7,989	4613	39.73
15D	32256	37727	31812	33,932	3,294	1902	10.62
16A	17432	32098	34435	27,988	9,216	5321	26.28

Extract ID	RFU 1	RFU 2	RFU 3	Sample Mean	stdev	SEM	% inhitbition
16B	371	351	348	357	13	7	99.06
16C	814	1196	1331	1,114	268	155	97.07
16D	-234	-316	-210	(253)	56	32	100.67
17A	38538	38232	38546	38,439	179	103	-1.25
17B	15287	18488	15934	16,570	1,693	977	56.35
17C	37689	37143	38221	37,684	539	311	0.73
17D	37034	38668	38478	38,060	894	516	-0.25
18A	37127	36912	36892	36,977	130	75	2.60
18B	3086	1152	1784	2,007	986	569	94.71
18C	1899	1102	1364	1,455	406	235	96.17
18D	2906	2587	1065	2,186	984	568	94.24
19A	40210	39860	39000	39,690	623	359	-4.55
19B	7004	11197	12617	10,273	2,918	1685	72.94
19C	33328	17303	19897	23,509	8,602	4966	38.07
19D	37551	38341	38754	38,215	611	353	-0.66
20A	36696	34419	35059	35,391	1,174	678	6.77
20B	33282	13523	12635	19,813	11,673	6739	47.81
20C	40346	40596	41348	40,763	522	301	-7.38
20D	39366	39577	40167	39,703	415	240	-4.58
21A	35021	33808	19700	29,510	8,517	4917	22.27
21B	284	331	40200	13,605	23,032	13298	64.16
21C	15139	5682	5449	8,757	5,528	3192	76.93
21D	38511	37625	39874	38,670	1,133	654	-1.86

Extract ID	RFU 1	RFU 2	RFU 3	Sample Mean	stdev	SEM	% inhitbition
Media Only	4409	4553	4562	4,508	86	50	NA
Untreated	41086	39185	39795	40,022	971	560	-
.5% DMSO	38424	40135	39160	39,240	858	496	1.95
Pentamidine	32	-44	-22	(11)	39	23	100.03
AN-A	39644	39339	38745	39,243	457	264	1.95
BE-A	39534	38485	38612	38,877	573	331	2.86
DE-A	39011	38446	38985	38,814	319	184	3.02
EU-A	38988	39045	37766	38,600	723	417	3.55
FL-A	38406	38806	39059	38,757	329	190	3.16
PA-A	39124	38316	40062	39,167	874	504	2.14
MA-A	-850	-883	-667	(800)	116	67	102.00
RI-A	38594	38705	37681	38,327	562	324	4.24
AN-B	38961	38685	38622	38,756	180	104	3.16

Extract ID	RFU 1	RFU 2	RFU 3	Sample Mean	stdev	SEM	% inhitbition
BE-B	38185	38903	40355	39,148	1,105	638	2.18
DE-B	177	203	606	329	241	139	99.18
EU-B	2670	2717	2244	2,544	261	150	93.64
FL-B	41877	41492	40039	41,136	969	560	-2.78
PA-B	40043	41078	39504	40,208	800	462	-0.47
MA-B	10095	10915	10638	10,549	417	241	73.64
RI-B	509	-403	-357	(84)	514	297	100.21
AN-C	38286	39271	38806	38,788	493	284	3.08
BE-C	35771	38712	37774	37,419	1,502	867	6.50
DE-C	38971	32249	36327	35,849	3,386	1955	10.43
EU-C	35239	38239	38106	37,195	1,695	979	7.06
FL-C	594	576	845	672	150	87	98.32
PA-C	39620	40666	41567	40,618	974	563	-1.49
MA-C	406	35	124	188	194	112	99.53
RI-C	-166	-389	-313	(289)	113	65	100.72
AN-D	1131	36314	39144	25,530	21,177	1222 7	36.21
BE-D	17562	37902	35909	30,458	11,212	6473	23.90
DE-D	384	3250	3949	2,528	1,889	1091	93.68
EU-D	7229	2427	1043	3,566	3,247	1874	91.09

Extract ID	RFU 1	RFU 2	RFU 3	Sample Mean	stdev	SEM	% inhitbition
Media Only	4933	4959	4990	4,961	29	16	NA
Untreated	34638	37671	36686	36,332	1,547	893	-
.5% DMSO	32545	33602	32928	33,025	535	309	9.10
Pentamidine	-28	-116	-122	(89)	53	30	100.24
FL-D	-7	-348	-250	(202)	176	101	100.56
PA-D	17925	12549	14228	14,901	2,750	1588	58.99
MA-D	-409	-532	-586	(509)	91	52	101.40
RI-D	-711	-867	-849	(809)	85	49	102.23
SB-1	182	204	212	199	16	9	99.45
SB-2	15067	16325	16126	15,839	676	390	56.40
SB-3	-298	-335	-304	(312)	20	11	100.86
SB-5	-526	-416	-476	(473)	55	32	101.30
SB-6	-496	-528	-461	(495)	34	19	101.36
CP-1	7261	7059	6328	6,883	491	283	81.06
CP-2	7145	8819	10411	8,792	1,633	943	75.80
CP-3	127	94	202	141	55	32	99.61
CP-4	11293	10031	10197	10,507	686	396	71.08

Extract ID	RFU 1	RFU 2	RFU 3	Sample Mean	stdev	SEM	% inhitbition
CP-5	15368	12367	13038	13,591	1,575	909	62.59
CP-6	14987	13754	13626	14,122	752	434	61.13
CT-1	16575	16303	17540	16,806	650	375	53.74
CT-2	18642	16893	18750	18,095	1,042	602	50.19
CT-3	353	600	1152	702	409	236	98.07
CT-4	1798	674	766	1,079	624	360	97.03
CT-5	20225	19587	19469	19,760	407	235	45.61
CT-6	16780	15608	9530	13,973	3,892	2247	61.54
MDZ-1	22074	33908	18740	24,907	7,971	4602	31.44
MDZ-2	21457	34878	20797	25,711	7,946	4588	29.23
MDZ-3	17080	18012	20124	18,405	1,560	900	49.34
MDZ-4	11650	14956	12826	13,144	1,676	968	63.82
MDZ-5	34669	34800	32405	33,958	1,347	777	6.53
MDZ-6	32313	34278	20120	28,904	7,670	4428	20.44
LP-1	3244	18006	13809	11,686	7,606	4392	67.83

Extract ID	RFU 1	RFU 2	RFU 3	Sample Mean	stdev	SEM	% inhitbition
Media Only	5084	4971	5211	5,089	120	69	NA
Untreated	16502	19845	15403	17,250	2,314	1336	-
.5% DMSO	12888	15081	11798	13,256	1,672	965	23.16
Pentamidine	-195	-122	-352	(223)	118	68	101.29
LP-3	7913	9179	10590	9,227	1,339	773	46.51
LP-4	11191	11879	11793	11,621	375	216	32.63
LP-5	10700	12214	11003	11,306	801	463	34.46
LP-6	1578	3643	1814	2,345	1,130	653	86.41

Appendix III

High Throughput Screen of Plant Extracts Against L6 Cells

Extract ID	RFU 1	RFU 2	RFU 3	Sample Mean	stdev	SEM	% inhitbition
Untreated	16,265	19,019	20,232	18,505	2,033	1,174	-
10% DMSO	21,070	20,019	20,357	20,482	537	310	(10.68)
10& EtOH	19,975	22,280	21,752	21,336	1,208	697	(15.29)
10% Water	19,905	20,395	21,492	20,597	813	469	(11.30)
PPT	2,082	2,114	2,533	2,243	252	145	87.88
1A	17,966	17,773	19,106	18,282	720	416	1.21
1B	12,904	13,998	14,165	13,689	685	395	26.03
1C	202	261	3,093	1,185	1,652	954	93.59
1D	10,790	9,128	9,941	9,953	831	480	46.22
2A	18,137	17,819	15,617	17,191	1,372	792	7.10
2B	18,839	17,851	17,690	18,127	622	359	2.05
2C	13,983	17,913	15,427	15,774	1,988	1,148	14.76
2D	22	58	108	63	43	25	99.66
3A	17,862	18,702	17,844	18,136	490	283	2.00
3B	12,396	15,091	12,018	13,168	1,676	968	28.84

Extract ID	RFU 1	RFU 2	RFU 3	Sample Mean	stdev	SEM	% inhitbition
Media Only	506	502	505	504	2	1	NA
Untreated	16,790	16,151	17,335	16,759	593	342	-
10% DMSO	18,031	18,951	18,139	18,374	503	290	(9.64)
10& EtOH	15,009	19,116	14,235	16,120	2,623	1,515	3.81
10% Water	17,693	18,995	16,971	17,886	1,026	592	(6.73)
PPT	2,869	3,869	3,046	3,261	534	308	80.54
1A	6,923	2,342	1,192	3,486	3,032	1,750	79.20
2B	15	(12)	(11)	(3)	15	9	100.02
3C	9,117	7,747	7,740	8,201	793	458	51.06
3D	11,469	12,808	12,462	12,246	695	401	26.93
4A	6,744	13,362	11,497	10,534	3,412	1,970	37.14
4B	11,275	13,971	13,934	13,060	1,546	893	22.07
4C	15,270	14,293	14,583	14,715	502	290	12.19
4D	13,843	11,689	13,134	12,889	1,098	634	23.09
5A	16,376	18,280	18,636	17,764	1,215	702	(6.00)
5B	13,535	13,587	13,926	13,683	212	123	18.35
5C	15,748	13,348	12,283	13,793	1,775	1,025	17.70
5D	14,807	10,185	12,343	12,445	2,313	1,335	25.74
6A	20,201	16,781	17,119	18,034	1,885	1,088	(7.61)
6B	6,968	8,616	10,226	8,603	1,629	941	48.66
6C	13,703	12,038	13,595	13,112	932	538	21.76
6D	14,822	13,727	14,300	14,283	548	316	14.77
7A	17,634	17,635	17,732	17,667	56	33	(5.42)

Extract ID	RFU 1	RFU 2	RFU 3	Sample Mean	stdev	SEM	% inhitbition
7B	5,341	6,047	7,770	6,386	1,249	721	61.89
7C	13,774	13,784	12,437	13,332	775	447	20.45
7D	14,234	13,616	10,574	12,808	1,959	1,131	23.57
8B	14,399	15,146	10,775	13,440	2,338	1,350	19.80

Extract ID	RFU 1	RFU 2	RFU 3	Sample Mean	stdev	SEM	% inhitbition
No Cells	507	516	515	513	5	3	NA
Untreated	16364	19233	21695	19097	2668	1,540	-
10% DMSO	19640	20806	21823	20756	1092	631	-8.69
10% Ethanol	19369	20515	21818	20567	1225	707	-7.70
10% Water	20462	19294	21408	20388	1059	611	-6.76
PPT	3382	3948	3376	3569	329	190	81.31
8C	12828	13990	14781	13866	982	567	27.39
8D	9754	10292	12606	10884	1515	875	43.01
9A	21240	21858	21098	21399	404	233	-12.05
9B	1560	3393	2684	2546	924	534	86.67
9C	8971	10335	13765	11024	2470	1,426	42.28
9D	15753	19999	20782	18845	2706	1,562	1.32
10A	12530	12856	15843	13743	1826	1,054	28.04
10B	48	72	41	54	16	9	99.72
10C	17061	17547	21346	18651	2346	1,355	2.34
10D	10900	10983	14696	12193	2168	1,252	36.15
11A	22465	20599	18801	20622	1832	1,058	-7.98
11B	7574	10135	15021	10910	3784	2,184	42.87
11C	23	20	76	40	32	18	99.79
11D	10400	9771	10285	10152	335	193	46.84
12A	22371	20116	21896	21461	1189	686	-12.38
12B	14820	18351	21429	18200	3307	1,909	4.70
12C	17000	18285	20805	18697	1936	1,118	2.10
12D	12337	12351	16661	13783	2492	1,439	27.83
13A	12608	18875	12372	14618	3688	2,129	23.45
13B	2434	4640	3677	3584	1106	639	81.23
13C	13384	15524	14467	14458	1070	618	24.29
13D	14273	18111	13293	15226	2546	1,470	20.27
14A	13603	19188	13397	15396	3286	1,897	19.38
14B	16639	17757	14298	16231	1765	1,019	15.01
14C	14983	16707	13473	15054	1618	934	21.17
14D	12184	13494	12538	12739	678	391	33.30

Extract ID	RFU 1	RFU 2	RFU 3	Sample Mean	stdev	SEM	% inhitbition
No Cells	462	474	466	467	6	4	NA
Untreated	18337	18207	19140	18561	505	292	-
10% DMSO	19994	19751	21767	20504	1101	635	-10
PPT	2910	2950	3236	3032	178	103	84
15A	20068	22738	21666	21491	1344	776	-15.78
15B	3421	4623	4832	4292	762	440	76.88
15C	13987	15473	13477	14312	1037	599	22.89
15D	15078	14986	15398	15154	216	125	18.36
16A	17272	18107	21216	18865	2078	1,200	-1.64
16B	220	722	99	347	330	191	98.13
16C	9331	10855	8303	9496	1284	741	48.84
16D	5826	3823	3900	4516	1135	655	75.67
17A	22195	22517	23046	22586	430	248	-21.68
17B	16476	18148	18404	17676	1047	605	4.77
17C	16099	17115	16329	16514	533	308	11.03
17D	13172	14679	13498	13783	793	458	25.74
18A	13864	16073	15717	15218	1186	685	18.01
18B	1440	1687	1952	1693	256	148	90.88
18C	10478	11411	11633	11174	613	354	39.80
18D	16494	15355	14127	15325	1184	683	17.43
19A	22075	20678	18848	20534	1618	934	-10.63
19B	17644	14989	16940	16524	1375	794	10.97
19C	15623	13901	15566	15030	978	565	19.03
19D	16391	13809	14479	14893	1340	774	19.76
20A	17496	14801	13098	15132	2218	1,280	18.48
20B	5655	8683	5259	6532	1873	1,081	64.81
20C	18236	16454	13616	16102	2330	1,345	13.25
20D	17889	17811	13711	16470	2390	1,380	11.27
21A	19640	19264	16544	18483	1689	975	0.42
21B	47	56	417	173	211	122	99.07
21C	10148	11409	9485	10347	977	564	44.25
21D	13937	15332	11962	13744	1693	978	25.96

Extract ID	RFU 1	RFU 2	RFU 3	Sample Mean	stdev	SEM	% inhitbition
No Cells	479	463	455	466	12	7	NA
Untreated	20333	21533	19923	20596	837	483	-
10% DMSO	22098	23741	22739	22859	828	478	-10.99
PPT	2810	2928	2747	2828	92	53	86.27
AN-A	21754	21790	22476	22007	407	235	-6.85
BE-A	21576	21798	21467	21614	169	97	-4.94

Extract ID	RFU 1	RFU 2	RFU 3	Sample Mean	stdev	SEM	% inhitbition
DE-A	17423	22164	21493	20360	2566	1,481	1.15
EA-A	18756	18739	19483	18993	425	245	7.79
FL-A	16206	15411	19846	17154	2365	1,365	16.71
PA-A	20869	20717	19383	20323	818	472	1.33
MA-A	17513	16819	15219	16517	1176	679	19.81
RI-A	19996	20263	19512	19924	381	220	3.27
AN-B	22325	22740	22136	22400	309	178	-8.76
BE-B	21552	21961	22600	22038	528	305	-7.00
DE-B	21598	21956	21284	21613	336	194	-4.93
EA-B	20447	19463	19394	19768	589	340	4.02
FL-B	21822	20350	20428	20867	828	478	-1.31
PA-B	22735	21994	21370	22033	683	395	-6.98
MA-B	21803	21570	21839	21737	146	84	-5.54
RI-B	22172	20968	21755	21632	611	353	-5.03
AN-C	20802	20217	20844	20621	351	202	-0.12
BE-C	19930	20557	20569	20352	366	211	1.19
DE-C	20651	19308	19996	19985	672	388	2.97
EA-C	20875	18140	18483	19166	1490	860	6.94
FL-C	2325	2176	3760	2754	875	505	86.63
PA-C	16597	14707	14977	15427	1022	590	25.10
MA-C	8936	7759	7155	7950	906	523	61.40
RI-C	15890	15216	14924	15343	495	286	25.50
AN-D	16985	17765	14150	16300	1902	1,098	20.86
BE-D	10028	11570	10015	10538	894	516	48.84
DE-D	13759	15057	9453	12756	2933	1,694	38.07
EA-D	16545	16331	12690	15189	2167	1,251	26.26

Extract ID	RFU 1	RFU 2	RFU 3	Sample Mean	stdev	SEM	% inhitbition
No Cells	710	701	690	700	10	6	NA
Untreated	18867	22540	21444	20950	1886	1,089	-
10% DMSO	22316	21455	21089	21620	630	364	-3.20
PPT	2830	3904	7241	4658	2300	1,328	77.76
FL-D	802	1281	1788	1290	493	285	93.84
PA-D	21347	21339	21213	21300	75	43	-1.67
MA-D	557	1087	2091	1245	779	450	94.06
RI-D	17153	19548	16925	17875	1453	839	14.68
SB-1	18626	18633	16347	17869	1318	761	14.71
SB-2	23401	23578	22975	23318	310	179	-11.30
SB-3	22050	22576	21121	21916	737	425	-4.61
SB-5	17155	16979	17811	17315	438	253	17.35

Extract ID	RFU 1	RFU 2	RFU 3	Sample Mean	stdev	SEM	% inhitbition
SB-6	22115	21449	20948	21504	585	338	-2.64
CP-1	20291	17883	17990	18721	1360	785	10.64
CP-2	22825	22180	20509	21838	1195	690	-4.24
CP-3	15089	16374	15301	15588	689	398	25.60
CP-4	14280	18506	20635	17807	3235	1,868	15.00
CP-5	20289	18394	20199	19627	1069	617	6.31
CP-6	19548	21059	18608	19738	1237	714	5.79
CT-1	20298	21352	20683	20778	533	308	0.82
CT-2	20055	21427	21481	20988	808	467	-0.18
CT-3	11774	9675	12904	11451	1639	946	45.34
CT-4	4063	3669	4914	4215	636	367	79.88
CT-5	20572	17999	19848	19473	1327	766	7.05
CT-6	17525	18779	18729	18344	710	410	12.44
MDZ-1	8378	8183	6122	7561	1250	722	63.91
MDZ-2	29852	16829	12955	19879	8852	5,111	5.12
MDZ-3	24012	21592	19104	21569	2454	1,417	-2.95
MDZ-4	11255	12929	10242	11475	1357	783	45.23
MDZ-5	1858	2009	2277	2048	212	123	90.22
MDZ-6	19536	21505	19375	20139	1186	685	3.87
LP-1	20454	19200	19468	19707	660	381	5.93

Extract ID	RFU 1	RFU 2	RFU 3	Sample Mean	stdev	SEM	% inhitbition
No Cells	705	695	700	700	5	3	NA
Untreated	21312	20242	20619	20724	543	313	-
10% DMSO	21029	19598	20617	20415	737	425	1.49
PPT	4567	3782	2894	3748	837	483	81.92
LP-3	19537	22593	20218	20783	1604	926	-0.28
LP-4	22693	21158	20773	21541	1016	586	-3.94
LP-5	21580	18577	18434	19530	1777	1,026	5.76
LP-6	17578	17927	15930	17145	1067	616	17.27