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DEVELOPMENT OF MICROSCALE ASSAYS TO SCREEN FOR NOVEL ANTHELMINTIC DRUGS

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DEVELOPMENT OF MICROSCALE ASSAYS TO SCREEN FOR NOVEL ANTHELMINTIC DRUGS

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ABSTRACT

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Parasitic nematodes are beginning to show resistance to the most commonly used classes of anthelmintic drugs at an alarming rate. As the number of documented cases of resistance increases, people are becoming more interested in identifying new sources of anthelmintic drugs. Since the emergence of popular anthelmintics in the 1980s, there has been little effort put into the development of new drugs for use against helminths. Recently, a few studies have investigated new sources for anthelmintics, including one that discovered a whole new class of compounds that showed anthelmintic activity against resistant nematode species. Previous studies used high volume assays which require milliliter amounts of the compounds being screened for anthelmintic abilities. The goal of this study was to develop new screening processes for identifying anthelmintic activity in naturally derived compounds using microliter volumes of the test compound solutions. Two microscale assays were developed using the nematode *Caenorhabditis elegans* as a model system: one looking for the immediate effects on motility and the other investigating the effects of each compound on development and fecundity of the worms. The assays developed in this project were used to identify several compounds of interest that could be further investigated as potential anthelmintic drugs.

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INTRODUCTION

The Global Threat of Animal Parasitic Nematodes

Parasitic nematodes, also known as helminths, are a major cause of concern for people all over the world. These parasites can cause significant health problems in humans and animals and also can result in great financial losses by harming food crops and food-producing animals. Sheep and goat producing countries in Asia and Africa, for example, spend more than 100 million dollars each year treating livestock infected by helminths (Waller & Chandrawathani, 2005).

There are many parasitic nematode species that currently concern the global health community. Although parasites most frequently infect people living in small villages in Africa or South America, human parasitic nematodes are found in the warmer regions of almost every continent (CDC, 2008). Parasites are known for their complex life cycles that often include multiple hosts or the re-infection of a single host (CDC, 2008).

Some of the more common species known to infect humans include *Onchocerca volvulus* (Kenney, 1973; Yamaguchi, 1981), *Necator americanus* (Yamaguchi, 1981), *Ascaris duodenale* (Yamaguchi, 1981), and *Ascaris lumbricoides* (Kenney, 1973; Yamaguchi, 1981). *Onchocerca volvulus* is transmitted by the black fly (WHO, 2008) and causes River Blindness, a disease resulting in skin deformities and loss of sight caused by the accumulation of worms within the host (Kenney, 1973; Yamaguchi, 1981).

Necator americanus and *Ascaris duodenale* are hookworms that prevent normal nutrient absorption and distribution in their human hosts by consuming blood and damaging the small intestine (Yamaguchi, 1981). They spread via skin contact by the infectious larval stage of the parasite, usually found in feces (CDC, 2008). The most common helminth, *A. lumbricoides*, has been diagnosed in people across the world, with the majority of cases coming from places with poor sanitation practices (Yamaguchi, 1981). People infected with *A. lumbricoides* experience digestive and respiratory complications (Kenney, 1973; Yamaguchi, 1981). The parasite is contracted when a person ingests feces containing the nematode's eggs (CDC, 2008).

Many parasitic nematodes can cause similar issues in pets and livestock. Infected animals can become costly to their owners in the form of treatment, loss of products (e.g. meat, milk, wool, etc.) from animals too weak or sick to produce, or death of the animal. Like their human-infecting relatives, pet and livestock nematode parasites also can be found virtually anywhere on the planet and have complex life cycles (Urquhart et al., 1996; Boreham and Atwell, 1988; Larsen, 2007). Most of these helminths cause digestive and circulatory system problems in infected animals (Urquhart et al., 1996; Boreham & Atwell, 1988). Some of the more common nematode parasites of livestock and pets include *Dirofilaria immitis*, the parasite responsible for heartworm disease (a buildup of *D. immitis* worms causing the blockage of blood vessels in the infected animal) in cats and dogs (Boreham & Atwell, 1988), *Haemonchus contortus*, an extremely common parasite in farm ruminants that latches onto the animal's digestive tract and feeds on blood vessels causing mild to severe anemia (Urquhart et al., 1996),

and *Ostertagia ostertagi*, a parasite of cattle known to cause digestive problems and death in their hosts (Urquhart et al., 1996).

The Dangers of Plant Parasitic Nematodes

Plant parasitic nematode species comprise 15% of all nematode species (Perry & Moens, 2006). Like their animal infecting relatives, plant parasitic nematodes can be found all over the world in almost any climate (Norton, 1978). These parasite species are physically very similar to nematodes that infect animals except that they possess a stylet structure specially designed to penetrate the plant's cell walls (Chandniwalla, 1996). The parasites feed almost exclusively on the roots of plants and are often found in high concentrations near vulnerable plants because they are capable of moving only short distances on their own (Chandniwalla, 1996). While many different types of plants are susceptible to nematode infections, the parasites themselves are often specific to one kind of host plant (Chandniwalla, 1996).

Plant parasitic nematodes fall into two main types: the endoparasites, including the root-knot and spiral nematodes, which enter the root of the plant and feed on internal structures, and the ectoparasites, including the ring and stubby-root nematodes, which feed on the plant's roots from the outside (Chandniwalla, 1996). As they eat, the nematodes introduce an enzyme-containing saliva into the cells of the plant's root which breaks down structures within the root cells (Chandniwalla, 1996). This saliva causes extensive damage to the plant's root system resulting in the plant having trouble transporting water and nutrients from the roots to the rest of the plant and leaving the plant more susceptible to infection by bacterial and fungal parasites (Chandniwalla, 1996;

Norton, 1978). An infected plant will often show signs of root cell death, stunted growth, low or poor quality yields, and will wilt more than a normal plant in hot or dry weather (Chandniwalla, 1996).

Plant parasitic nematode species have been known to infect many food crops like corn, beets, potatoes, soybeans, and fruit trees, as well as non-food plants, including turf and other landscaping plants (Perry & Moens, 2006). Because these parasites infect non-motile hosts and are not able to travel large distances on their own, they are spread primarily through human activities such as the transport of parasite-laden soil on unwashed farm equipment and through irrigation channels (Chandniwalla, 1996). There are several chemicals on the market used to treat parasitic nematode infections in plants including sodium methyldithiocarbamate, methyl bromide, ethylene dibromide, and dimethyltetrahydrothiadiazinethione (Chandniwalla, 1996). These chemicals are broad spectrum pesticides, meaning that they kill not only nematodes, but many other kinds of pests including insects, fungi, and weeds. They come as liquids or pellets and are applied before or after planting (depending on the chemical) and work by releasing toxic gasses that diffuse through the soil, a process that can be harmful to people, pets, and livestock, along with the intended parasite targets (Chandniwalla, 1996). To reduce the spread of these gasses into the air and to keep them at high concentrations near the infected plants plastic coverings are secured over treated areas. The exact mechanism of action for these chemicals is not fully understood, but it is believed that they may act on the nervous system of the parasite (Chandniwalla, 1996). Because these chemicals are very dangerous to humans and animals, a number of practices have developed to reduce the spread of nematode infection (Chandniwalla, 1996). Preventative measures include

thoroughly cleaning farm equipment before moving it to a new area, rotating crops to plant a non-susceptible species, and genetically engineering parasite resistant plants (Chandniwalla, 1996; Perry & Moens, 2006).

Available Anthelmintic Agents and Their Modes of Action

Due to the prevalence of infection in plants, humans, and other animals by parasitic nematodes there is a great need for anthelmintic drugs to treat these infections. Currently, there are only a few effective anthelmintic agents on the market for treating animal-parasitizing helminth species (Van Zeveren et al., 2007). These include broad spectrum anthelmintics, which work on a wide variety of parasitic species, and some narrow spectrum drugs that act on only a few specific species. Three main families of chemicals are used most commonly to treat livestock and human parasites: benzimidazoles, imidazothiazoles & tetrahydropyrimidines, and macrocyclic lactones (Anderson & Waller, 1985). All three work on several species of parasites and have different mechanisms of action (Table 1).

Table 1. Summary of the major anthelmintic drug classes.

Drug Class	Mechanism of Action	Example Drug	Used to treat
Benzimidazoles	Bind β-tubulin preventing microtubule formation in cells.	Albendazole	*Ascaris lumbricoides, Necator americanus, Ascaris duodenale*
Imidazothiazoles & Tetrahydropyrimidines	Bind to nicotinic receptors causing erratic signaling and muscle paralysis.	Pyrantel	*Ostertagia ostertagi, Ascaris lumbricoides, Necator americanus, Ascaris duodenale*
Macrocyclic Lactones	Keep chloride channels open by binding subunits of channel. Excess chloride causes muscle paralysis.	Ivermectin	*Onchocerca volvulus, Dirofilaria immitis*

The benzimidazoles represent a large number of currently used anthelmintic drugs. Albendazole is the most common benzimidazole used to treat human helminth infections (Katzung et al., 2009). These drugs work by binding to β-tubulin within cells and preventing formation of microtubules, a key component of the cytoskeleton (Prichard, 2005). The parasite's host is mostly unaffected by the benzimidazoles because the drug has a higher affinity for invertebrate β-tubulin, which has a slightly different protein sequence than mammalian β-tubulin (Waller, 2007). Without microtubules, important cellular processes like cell division and the transport of molecules and organelles throughout the cell can no longer occur, resulting in the death of the cells and ultimayely the entire organism. The dead parasite can then be destroyed by the host's natural immune response. Because they are so effective, these drugs have been used very frequently to treat infected cattle, sheep, and horses.

The next major class of broad spectrum anthelmintics, the imidazothiazoles & tetrahydropyrimidines, is represented by two drugs: levamisole and pyrantel. Most often used to treat parasites in the gastrointestinal tract, these drugs are effective against both young and adult parasites, but not against young worms in stages of developmental arrest (Merck & Co., 2008). These drugs work by binding to nicotinic receptors at neuromuscular junctions and causing the receptor to fire uncontrollably (Prichard, 2005). At low doses, the host will be unaffected by these drugs, but increasing the dose results in side effects in the parasite's host (Waller, 2007). The improper firing of these receptors prevents muscle function and results in paralysis. This paralysis of the muscles prevents the nematode from feeding, eventually leading to the death of the parasite. The host organism's immune system can then destroy the remains of the parasites.

Macrocyclic lactones, the final class of broad spectrum anthelmintics, contain the most popular group of anthelmintic drugs, the avermectins. They are derived from the bacterium *Streptomyces avermitilis* (Burg et al., 1978) and are very effective against young parasitic nematodes, even at low doses (Merck & Co., 2008). The macrocyclic lactones are commonly used in humans and livestock to treat infections in several parts of the body (Eng et al., 2006). The drugs force chloride channels to stay open by binding to the subunits of glutamate-aminobutyrate gated and γ-aminobutyrate gated channels, resulting in muscle paralysis (Prichard, 2005). The host organism is unaffected because they only possess this type of chloride channels in their central nervous system where the drug cannot reach (Waller, 2007). Ivermectin is the most commonly used avermectin. Ivermectin is very effective against many parasitic nematode species that threaten people in warmer climates, so it is the drug of choice for healthcare providers and livestock producers in those regions (Moncayo et al., 2008).

Emerging Drug Resistance to Anthelmintics

The excessive and inappropriate use of broad-spectrum anthelmintics, especially the benzimidazoles and avermectins, has resulted in a rapid increase in the number and incidence of drug resistant nematode strains (Geerts & Gryseels, 2001; Lochnit et al., 2005; Merck & Co., 2008; Van Zeveren et al., 2007). Although resistance has been documented in human helminths, it is much more prevalent in livestock-infecting nematodes. Drug resistance refers to the reduced effectiveness of a drug on a parasite target that was once susceptible to the drug (Sangster & Gill, 1999). This phenomenon occurs when a parasite target experiences a genetic change that results in an increased

ability to survive in the presence of a substance that was once toxic to the organism (Bowman et al., 2005). The individuals possessing the genes that protect against the drug will survive and thrive in the environment, whereas competition is reduced by the drug. Drug resistance is more likely to occur in parasites with shorter, less complex life cycles (Sangster & Gill, 1999). The possibility of developing drug resistance also depends on parasite load, how much drug is used, and whether or not it is administered properly (Bowman et al., 2005). Anthelmintic drug resistance was first observed in the parasites of sheep and horses against some drugs in the benzimidazole family (Prichard, 2005). Since then, many more instances of drug resistance have been documented in ruminant animals and horses, both within the benzimidazole family and in the other broad spectrum drug groups (Van Wyk et al., 1997). It is suspected that the drug resistance currently observed in nematodes infecting livestock is simply foreshadowing a future problem with human-infecting parasites (Keiser & Utzinger, 2008). A recent and dramatic increase in helminths resistant to ivermectin, a broad spectrum anthelmintic, is concerning to both veterinary and human healthcare providers because it is frequently used in both populations (Geerts & Gryseels, 2001).

The Quest for New Anthelmintic Drugs

Because of the growing resistance to popular and widely used anthelmintics like ivermectin, the identification of new pharmacological agents with anthelmintic activity is essential. Due to the dangers and complexities of working with actual parasites in the research laboratory, scientists rely on model organisms to study them. One commonly used model for helminths is the soil-dwelling nematode, *Caenorhabditis elegans*, a 1 mm

long, non-parasitic nematode that develops from embryo to adult in three days. The worm goes through four larval stages (L1-L4) before maturing into adult worms capable of producing embryos. The species has a short lifespan of approximately two weeks. They may be developed on an agar bed with a bacterial lawn for food, so they are much simpler to maintain than parasitic nematodes (Stiernagle, 2006). *Caenorhabditis elegans* worms are highly similar to helminths in their neuromuscular anatomy, and they are sensitive to existing anthelmintics at the same doses as parasitic nematodes (Holden-Dye & Walker, 2007). In fact, the molecular mechanisms by which current anthelmintics act was elucidated using *C. elegans* as a model (Holden-Dye & Walker, 2007). Because of this, several studies on anthelmintic drugs, including studies on resistance to ivermectin, have been performed using *C. elegans* as a parasite model for more than twenty years (Dent et al., 1999).

A new anthelmintic has not been brought to market in over 25 years. Only recently have scientists begun looking for potential new anthelmintic agents because of the increasing problem of drug resistance. Notably, one new family of anthelmintic compounds, the amino-acetonitrile derivatives (AADs), was reported (Kaminsky et al., 2008). The AADs are low molecular weight compounds consisting of an amino-acetonitrile core surrounded by different synthetically added aroyl and aryloxy side chains (Kaminsky et al., 2008). More than 600 AADs were tested against the ruminant parasites *Haemonchus contortus* and *Trichostrongylus colubriformis* in an *in vitro* larval development assay. Compounds that successfully restricted parasite growth were later tested in rodents that had been infected with the parasites and in a smaller number of cattle and sheep (Kaminsky et al., 2008). These researchers also showed that wild-type

C. elegans were negatively affected by specific AADs, and that they exhibited behavioral and developmental problems distinct from the defects observed in the presence of traditional anthelmintics. Interestingly, mutant strains of *C. elegans* that are resistant to existing anthelmintics were still affected by exposure to at least one AAD. By the end of the study, one AAD compound was identified that was able to eliminate all parasite strains tested, including the drug resistant ones, at low doses and without having any severe toxic effects on the parasite's host organism (Kaminsky et al., 2008). The power of *C. elegans* as a genetic model system is being exploited to pursue the molecular mechanism by which AADs act. Early evidence suggests that AADs interfere with a nematode specific subunit of the nicotinic acetylcholine receptor, known as ACR-23, resulting in the death of the organism (Kaminsky et al., 2008).

With drug resistance increasing at an alarming rate, additional anthelmintic agents will be needed, and natural products offer vast chemical diversity and potential for therapeutic use. Plants and fungi have been used to treat disease for thousands of years (Wang et al., 2007), and approximately half of the drugs that have entered the market in the last twenty years have come from natural sources (Dunkel et al., 2005). Now that the technology exists to identify the active molecules in medicinal plants and fungi, people are becoming increasingly interested in identifying these compounds and producing them for use as medicines and health supplements. Stilbene compounds are one class of biologically active molecules currently being researched. Stilbenes are found in many kinds of plants, and they are often released in response to stress caused by bacterial or fungal infection (Gorham, 1995). One stilbene that has received attention in recent years for its potential health benefits is resveratrol (Gorham, 1995; Likhtenshtein, 2010;

Dewick, 2002). Resveratrol is found at high levels in grapes and lower levels in other plants, including cranberries (Dewick, 2002; Likhtenshtein, 2010). Experiments using fruit flies, worms, mice, and yeast have demonstrated that resveratrol exhibits antioxidant and anticoagulant activities, and inhibits tumor formation, but none of these benefits have been confirmed in humans (Gorham, 1995; Likhtenshtein, 2010; Dewick, 2002). There are currently many studies in progress attempting to discover whether or not these benefits of resveratrol will exist in humans (Likhtenshtein, 2010).

A team of multi-disciplinary researchers at the University of Wisconsin-La Crosse have created a library of approximately three-hundred crude fungal extracts and over one-hundred pure synthetic compounds that are being screened for different biological activities. The fungal samples were collected and processed from the fruiting bodies of wild mushroms. Fruiting bodies are structures made by fungi that produce and disperse spores necessary for reproduction and serve as a place for the processing and storage of fungal waste products absorbed by the fungi (Moore-Landecker, 1996; Deshmukh & Rai, 2005). Due to its role in chemical processing within the fungi, the fruiting body contains many unique compounds that are not commonly found elsewhere (Moore-Landecker, 1996). The goal of this project was to screen this library of fungal extracts and synthetic stilbene derivatives for anthelmintic activity. Specifically, two microscale assays were designed to test the effects of each fungal extract or pure synthetic compound on the development, motility, and reproduction of the model organism, *C. elegans*.

SPECIFIC AIMS

Develop assays for screening fungal library extracts and synthetic compounds for effects on *C. elegans.*

1. The project designed a short-term assay that can be performed in a microtiter plate to screen *C. elegans* for paralysis or decreased motility. The assay allowed exposure to fungal extract or synthetic compound for a period of 12-16 hours.
    - All the available fungal extracts and all of the synthetic compounds deemed relevant based on their chemical structures were screened using this assay.
2. The project also designed a long-term assay that can be performed at microliter volumes to screen *C. elegans* for developmental arrest or sterility. The assay allowed the *C. elegans* to grow from L1 stage to adults (approximately four days in normal worms) in a liquid media containing the synthetic compound.
    - Synthetic derivatives deemed relevant based on their chemical structures were screened using this assay.

MATERIALS AND METHODS

Worm Cultures

The N2 (wild-type) strain of *C. elegans* was used in all assays. The worms were cultured on medium-sized Nematode Growth Medium (NGM) agar plates seeded with OP50 *Escherichia coli*. Two L4 stage hermaphrodite worms were picked from an original plate of N2 worms and placed on a new NGM worm plate and left for 3-4 days to

establish a new culture of approximately 100-150 worms. This process was repeated daily to prepare 4-6 plates of worms for use in the assays.

Preparation of Resuspended Worms for Motility Assays

Worms were collected from culture plates using a minimal salts buffer called M9 which contains monobasic potassium phosphate, dibasic sodium phosphate, sodium chloride, and 1 M magnesium sulfate combined in nanopure water (Stiernagle, 2006). The M9 was poured onto the plates and swirled to lift the worms. The buffer was then removed using a Pasteur pipette and placed in a 15 mL Falcon tube. The plate was then rinsed a second time to maximize the number of worms successfully suspended in the buffer. This process was repeated for each of the worm plates to be used in the assays. The tubes were inverted a few times to rinse debris off the worms and spun in a clinical centrifuge at the lowest speed setting for 1-2 minutes. The M9 was then drawn from the Falcon tube using a Pasteur pipette leaving the worm pellet. The tube was then refilled with M9, inverted a few times to rinse any remaining contaminants from the worms, and spun in the clinical centrifuge for another 1-2 minutes. The M9 was then removed, again leaving the worm pellet, and the tube was refilled with M9 to contain approximately 8 mL of M9 buffer with approximately 400-500 worms.

Motility Assay to Screen Fungal Extracts

The crude fungal extracts were prepared by crushing wild mushrooms, separating the smashed mushrooms into samples, and suspending each sample in dimethyl sulfoxide (DMSO) at concentrations of 256 mg/mL. Because the fungal extracts and synthetic compounds are suspended in DMSO, which is toxic to living organisms, a motility assay

was performed to expose worms to varying concentrations of DMSO and determine how much the nematodes could tolerate. The assay was run in microtiter plates, with each well containing a final volume of 200 µL. Wells were prepared by adding M9 buffer and DMSO to 100 µL of worm solution to create final DMSO concentrations ranging from 1-10% DMSO. Each concentration of DMSO was tested six times on the microtiter plate with wells containing approximately 30 worms. The plates were incubated at 20 °C overnight and viewed the next day with a dissecting microscope to see how many worms were alive and moving in each well. The total number of adult worms, along with the number of those adults that were motile, was counted.

To confirm that the *C. elegans* would respond to a known anthelmintic in a similar manner to parasitic nematodes, they were exposed to ivermectin, a known anthelmintic drug, at concentrations that elicited effects in previous studies (Holden-Dye & Walker, 2007). A 1 mg/mL ivermectin stock solution was made in 1 mL of DMSO, and this stock was further diluted to test four concentrations of ivermectin: 0.01 ng/ µL, 0.025 ng/ µL, 0.05 ng/ µL, and 0.1 ng/ µL. The assay was prepared in a microtiter plate with each well containing M9 buffer, resuspended worms, and ivermectin to a final volume of 200 µL. Each ivermectin treatment had six replicates on each microtiter plate. The plate was incubated overnight at 20 °C and viewed on a dissecting microscope to look for paralyzed worms in each well. Paralyzed worms are non-motile and form piles of straightened worms at the bottom of the test well. The total number of adults and the number of paralyzed adults was counted.

Fungal extracts were received in 40 µL aliquots at a standard concentration of 256 mg/mL in DMSO. Using microtiter plates, *C. elegans* were assayed for motility defects

ranging from decreased movement to complete paralysis. The extracts were prepared for testing by heating for ten minutes in a 55 °C water bath followed by centrifugation for one minute at maximum speed to pellet debris. Each well of the microtiter plate contained a total volume of 200 µL, which included 100 µL of resuspended worms in M9 buffer and 100 µL of fungal extract diluted in M9 buffer to the final concentrations corresponding to 1%, 2%, and 3% DMSO. Each plate had a positive control well containing ivermectin at 1 ng/µL and negative control wells with M9+DMSO at concentrations of 1-3% or M9 buffer only. The plates were incubated at 20 °C for 12-16 hours and were examined and worms scored for decreased movement, paralysis, or death. Each extract was screened twice.

Motility Assay to Screen Synthetic Compounds

Motility assays were performed on a series of molecules derived from a natural product isolated from a green plant that previously demonstrated interesting antimicrobial activites. The pure synthetic compounds were provided at a concentration of 10.24 µg/µL. They were tested in a set of serial dilutions beginning with 4 µL of the synthetic compound solution in 196 µL of M9 buffer (concentration approximately102.4 µg/mL). One-hundred microliters of this was removed and added to a second well containing 100 µL of M9 buffer to create a well that was half as concentrated as the original well. This process was repeated three times resulting in five different concentrations of the synthetic compounds. The wells were brought to a final volume of 200 µL by adding 100 µL of worm solution. Each plate contained the ivermectin (1ng/µL) positive control as well as M9+DMSO (same dilutions as the compounds tested), and M9 only negative controls.

The plates were incubated at 20 °C overnight and viewed on a dissecting microscope the next day to check for decreased movement, paralysis, and death. Each compound was screened in duplicate.

Preparation of Synchronized L1 Worms for Development Assay

To perform the developmental assay, a large number of worms at the same developmental stage were needed to ensure that variations in development during the assay were the result of the compounds being examined. To create a suspension of worms at the same stage in development, several plates of worms were bleached to harvest embryos from adult worms, as follows. Ten microliters of a 12% NaOH and 25% sodium hypochlorite (commercial bleach) bleaching solution was prepared by combining M9 buffer, 4M NaOH, and commercial bleach in a 15 mL falcon tube. After preparing the bleaching solution, worms were washed from five medium sized NGM plates, collected in a 15 mL falcon tube, and spun in a clinical centrifuge at 2000 rpm for 1-2 minutes. The supernatant was removed leaving the worms in 1-2 mL of M9. The bleaching solution was mixed using a vortex mixer and 6 mL was removed and added to the washed worms. The worms were suspended in the bleach solution for three minutes, with constant tapping on the lab bench to facilitate degradation. After three minutes, a small sample was removed from the falcon tube with a Pasteur pipette and checked for signs of degradation. Once degradation was observed, the bleached worms were immediately placed in a clinical centrifuge and spun at 2,000 rpm for one minute. The supernatant was removed and M9 was added to bring the volume to 10 mL. Worms were resuspended by inversion and centrifuged again at 2,000 rpm for one minute. Three more

washes with M9 buffer were carried out to remove all of the bleach solution. After the final wash, the bleached embryos were left in a microfuge tube with 1 mL of M9 buffer and incubated at 20 °C overnight to allow for all embryos to hatch. The newly hatched worms were arrested at the L1 stage because there was no food in the tube to allow developmental progress.

Developmental Assay

A developmental assay was performed on pure compounds from the synthetic library that previously showed activity in the motility assay. Three 5 mL overnight cultures of OP50 *E. coli* were separated into sterile microfuge tubes and microfuged at 12,000 rpm for three minutes to pellet the bacteria. The supernatant was removed and the bacterial pellets were added to a falcon tube. To bring the volume to 10 mL, M9 buffer was added to the falcon tube containing the bacterial pellets. Ampicillin and nystatin were added to the tube and allowed to sit for two hours, generating the "test media." Assays were performed in a covered microtiter plate. Each synthetic derivative was screened using the same serial dilutions as the synthetic derivate motility assay, with test media used to bring the samples to the final volume of 200 µL instead of M9 buffer. Every assay plate contained ivermectin, M9 only, and M9+DMSO control wells. For this assay, the ivermectin wells used a serial dilution with 1 ng/µL as the highest concentration. Ten microliters of L1s from the bleached worms were added to each test well. The plate was covered and incubated at 20 °C. The plates were checked daily for developmental progress. The developmental stage and activity level of each treatment were documented until the worms in the M9 only well reached adulthood and produced

viable offspring (four days). Treated wells were compared to the M9 only wells and any differences were noted.

RESULTS

Fungal Extract Activity Assay

Prior to conducting motility or developmental assays, the proper assay conditions and controls were determined. Because all of the fungal extracts and synthetic derivatives were dissolved in DMSO, it was necessary to establish the maximum concentration of DMSO that the worms could tolerate to prevent deleterious effects on the animals from the solvent alone. Worms were incubated overnight in 1-10% DMSO concentrations, and it was concluded that they could survive and maintain normal motility in DMSO levels at 3% or lower (Figure 1).

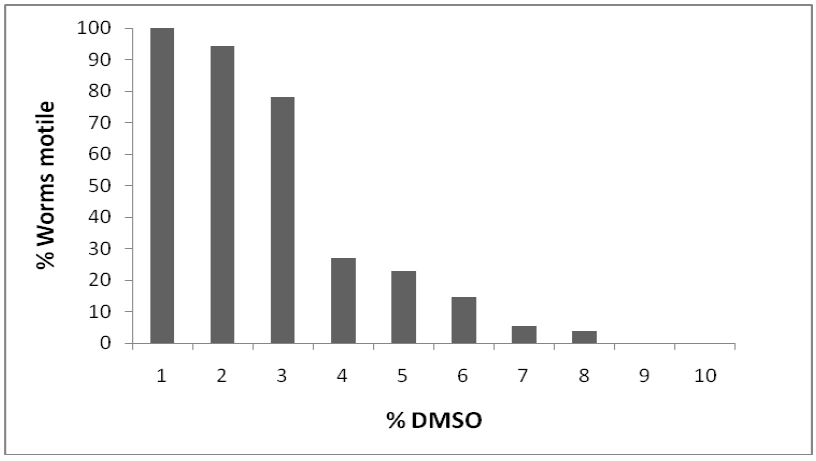

Figure 1. Percentage of worms motile after overnight exposure to various DMSO concentrations. The number of motile adult worms in each well and the total number of adult worms in each well was counted to determine the percentage of motile worms in each well after treatment. A sharp drop in survival was noted after DMSO concentrations exceeded 3%.

As a positive control, worms were exposed to ivermectin, which is a highly studied and commonly used anthelmintic agent known to affect the neuromuscular system of nematodes and cause paralysis. Animals were incubated in microtiter plates overnight with concentrations of ivermectin ranging from 0.1 ng/µL to 1 ng/µL. More than 90% of animals in each well were paralyzed (Figure 2) at the 0.05 ng/µL treatment, and all but a few worms were paralyzed in the 1 ng/µL treatment (Figure 3).

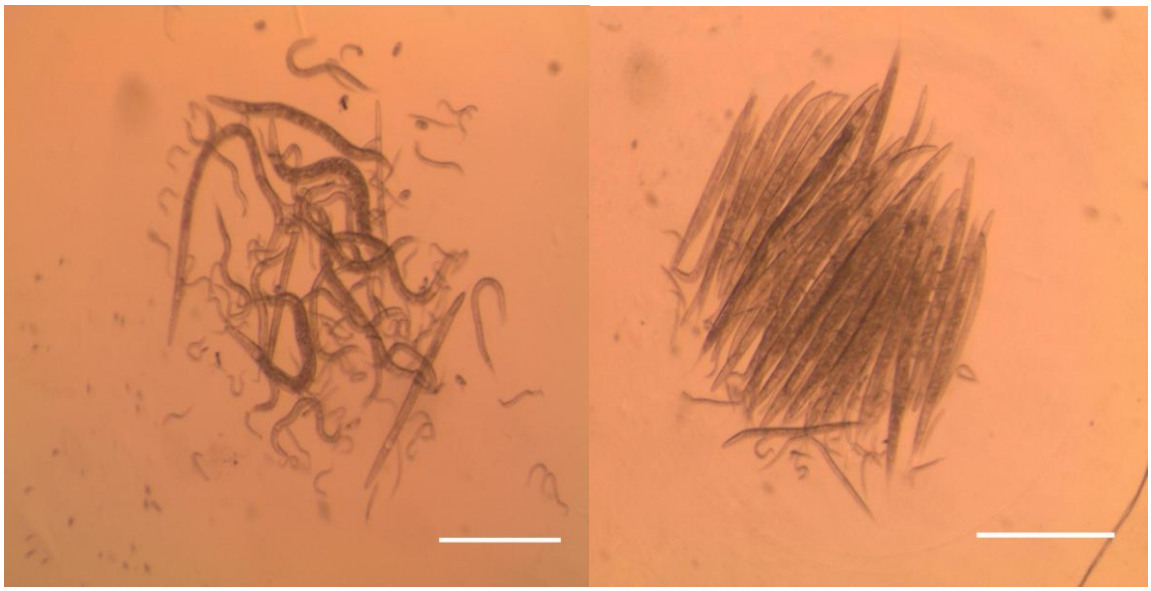

Figure 2. *Caenorhabditis elegans* in M9 Buffer (left) and after treatment with 1 ng/µL ivermectin (right). Worms in the M9 Buffer are unorganized and show muscle function. Most worms in the ivermectin treated well show the characteristic straightening associated with paralysis. Images taken with Leica DFC295 microscope camera (10X magnification). Scale bars = 500 µm.

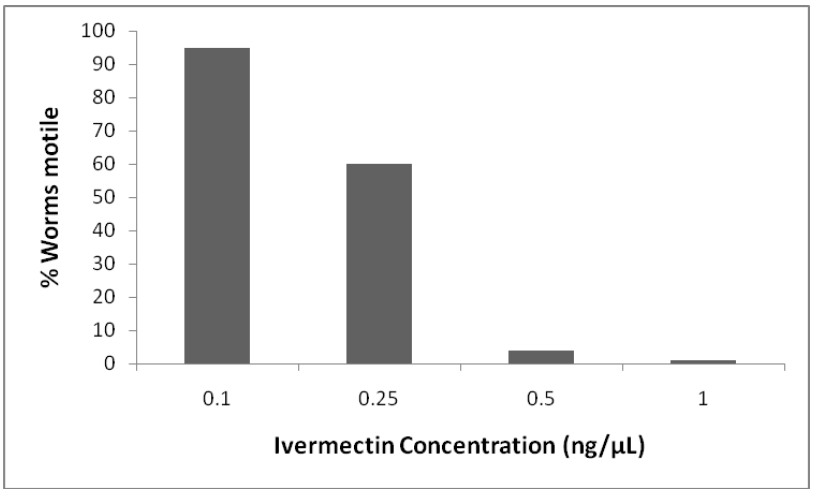

Figure 3. Percentage of worms motile after treatment with various concentrations of ivermectin. The number of motile worms and total number of worms were counted to determine the percentages for each treatment. Most worms were paralyzed after exposure to 0.5 ng/µl of the drug.

A total of 174 crude fungal extracts were screened in the motility assay (Table 2). The movement of animals was scored and classified as "normal" (-), "slow" (+), "very slow" (++) or "no movement/straightened" (+++). Animals determined to be "normal" moved in a manner similar to those in the M9 only control wells. "Slow" worms moved around, but not as vigorously as those in the M9 only wells, and these often showed signs of distress (jerky or uncoordinated movements). Animals exhibiting "very slow" movements only moved when agitated (tapping the plate or removing fluid from the well) and only moved the head or tail ends of their bodies. Worms that never moved, even when agitated, were classified in the "no movement/straightened" category. Of these extracts, six showed noticeable signs of activity (extracts F72, F181, F250, F283, F379, and F402). Extract F181 was the only one that showed strong activity (+++; no movement/straightened) in the worms at the lowest tested concentration.

Table 2. Crude extract motility assay data.

Extract	1/100	1/50	3/100	Extract	1/100	1/50	3/100	Extract	1/100	1/50	3/100
F9	-	+	+	F218	-	-	+	F374	-	++	+++
F11	-	-	++	F220	-	-	++	F375	-	-	+
F12	-	+	+	F222	-	-	+	F376	-	+	++
F13	-	-	++	F224	-	-	+	F378	-	+	+++
F19	-	-	-	F225	-	-	++	F379*	++	++	+++
F23	-	-	+	F226	-	-	+	F380	-	-	-
F24	-	+	+	F228	-	-	++	F381	-	++	+++
F31	-	-	+	F229	-	-	++	F382	-	-	+
F35	-	-	-	F231	-	+	++	F384	-	+	ND
F37	-	-	+	F233	-	-	+	F385	-	-	-
F42	-	+	++	F235	-	-	+	F386	-	-	-
F48	-	-	+	F238	-	-	-	F387	-	-	-
F59	++	++	+++	F241	-	-	+	F388	-	+	+
F60	-	+	++	F242	-	+	+	F389	-	+	+
F64	-	-	++	F243	-	-	+	F390	-	-	+
F68	-	+	+++	F244	-	-	+	F399	-	-	++
F69	+	ND	ND	F248	-	+	++	F400	-	-	+
F70	-	+	+	F249	-	+	++	F401	-	-	+
F72*	+	++	+++	F250*	+	+++	+++	F402*	++	++	+++
F89	+	+	++	F251	-	+	++	F403	-	+++	+++
F103	+	+	+	F252	-	-	+	F404	-	-	+
F107	-	+	++	F254	-	-	+	F405	-	-	+
F112	-	-	+	F255	-	-	+	F406	-	-	+
F125	-	-	++	F256	-	-	-	F407	-	+	+
F127	-	+	+	F259	-	-	+	F408	-	-	+
F129	-	-	+	F260	-	-	+	F409	-	-	+
F131	-	+	+	F263	ND	ND	ND	F419	-	+++	+++
F138	-	+	++	F264	-	-	-	F420	-	-	++
F139	-	-	+	F265	-	-	+	F422	-	+	++
F143	-	-	+	F266	-	-	+	F423	-	+	++
F145	-	-	-	F267	-	+	+	F424	-	+++	+++
F146	-	-	-	F268	-	-	-	F425	-	-	++
F147	-	ND	ND	F269	-	-	+	F426	-	-	-
F148	-	-	+	F270	+	+	+	F427	-	-	+
F150	-	+	+	F272	-	-	+	F429	-	+	++
F159	-	+	+	F274	-	+	++	F430	-	++	++
F163	-	+	+	F275	-	-	+	F431	-	+++	++
F164	-	+	+	F277	-	+	+	F432	-	-	+

Extract	1/100	1/50	3/100	Extract	1/100	1/50	3/100	Extract	1/100	1/50	3/100
F166	-	-	+	F278	-	+	++	F433	-	-	+
F167	-	-	++	F279	+	++	++	F434	-	-	++
F169	-	+	+	F280	-	-	+	F435	-	-	++
F173	-	-	+	F281	-	-	++	F437	-	-	-
F174	+	++	++	F282	+	++	++	F438	-	-	+
F175	-	-	+++	F283*	+	+++	+++	F439	-	-	-
F178	-	-	+++	F296	-	+	+	F440	-	-	-
F179	-	-	+++	F305	-	-	++	F441	-	-	-
F181*	+++	+++	+++	F311	-	+	+	F442	-	+	+
F186	+	+	+++	F326	-	-	+	F443	-	-	+
F187	-	-	+	F331	+	++	++	F444	-	-	++
F192	-	-	++	F335	+	ND	ND	F445	-	+	++
F193	-	+	+++	F351	-	-	+	F446	-	-	-
F195	-	-	-	F352	-	-	+	F447	-	-	+
F198	-	+	+++	F353	-	-	-	F448	-	-	-
F202	-	-	-	F364	+	++	+++	F449	-	-	-
F206	-	-	+	F365	-	-	+	F450	-	-	-
F212	-	-	+	F369	-	++	+++	F451	-	+	+
F213	-	+	+	F370	-	-	-	F452	-	-	-
F216	-	-	+	F373	-	-	-	F453	-	-	++
M9 + DMSO	-	-	+	Ive (1ng/µL)	+++	+++	+++	M9 Only	-	-	-

[1] Movements scored as normal (-), slow (+), very slow (++), or not motile/paralyzed (+++). ND=no data.
[2] M9 only, M9+DMSO (1-3% DMSO), and ivermectin (Ive; 1ng/µL) controls also included.
[*] Extracts marked with an asterisk consistently showed activity (distress, paralysis, and/or death) at 1% DMSO and/or 2% DMSO

Synthetic Derivatives Motility Assay

A total of 67 synthetic compounds were screened in the motility assay. The first 40 compounds examined were screened using the same methods as the fungal extracts assay, but it was determined that the concentrations were too high to provide useful results. The serial dilution protocol was used beginning with twelve compounds from the initial assay that appeared to show some activity. Twenty-seven additional structurally similar compounds were selected to screen using the new protocols (Table 3). The assay was scored using the same criteria as in the fungal extract motility assay (normal [-], slow [+], very slow [++], or not motile/paralyzed [+++]). Six of the compounds tested caused noticeably reduced motility, paralysis, or death in worms at the lowest concentrations tested (A9, A10, CL-5, SK-03-28F_2, SK-04-23, and SK-05-17). Of these, CL-5 and SK-05-17 were the most active, causing severe distress in worms at the lowest concentration tested.

Table 3. Synthetic derivative motility assay data.

Synthetic Compound	1/1600	1/800	1/400	1/200	1/100	Synthetic Compound	1/1600	1/800	1/400	1/200	1/100
A6	-	+	++	+++	+++	SK-04-57	-	-	-	-	+
A8	+	+	++	++	+++	SK-04-59F1	-	-	-	-	-
A9*	+	++	++	+++	+++	SK-05-01	-	-	-	-	+
A10*	+	++	+++	+++	+++	SK-05-02	-	-	+	+	+
CL-1	-	-	-	+++	+++	SK-05-03	-	-	-	+	+
CL-2	-	-	+++	+++	+++	SK-05-13	-	-	-	-	-
CL-3[#]	-	-	+	+++	+++	SK-05-14	-	-	-	+	++
CL-4	-	+	++	+++	+++	SK-05-15	-	-	-	-	-
CL-5*	++	+++	+++	+++	+++	SK-05-16	-	+	++	++	++
CL-6	-	-	+++	+++	+++	SK-05-17*	++	++	++	++	++
SK-03-28F2*	-	++	++	+++	+++	SK-05-21	-	-	++	+++	+++
SK-03-77	-	-	-	-	-	SK-05-22	-	+	++	+++	+++
SK-03-92[##]	-	-	-	-	+	SK-09-06	-	-	-	-	-
SK-04-03	-	+	+	++	++	SK-09-54	-	-	-	++	++
SK-04-08	+	+	++	+++	+++	SK-09-61	-	-	-	-	-
SK-04-22	-	-	+	+	++	VR-072209-01	-	-	-	-	-
SK-04-23*	+	++	++	++	++	VR-072209-02	-	-	-	-	-
SK-04-48	-	-	-	+	+	VR-072309-01	-	+	+++	+++	+++
SK-04-48F1	-	-	-	-	+++	VR-072309-02	-	-	-	-	-
SK-04-50	+	+	+	+	++	M9+DMSO	-	-	-	-	-
Ive (1ng/µL)	+++	+++	+++	+++	+++	M9 Only	-	-	-	-	-

[1]Movements scored as normal (-), slow (+), very slow (++), or not motile/paralyzed (+++).
[2] M9 only, M9+DMSO, and ivermectin (Ive; 1ng/µL) controls also included.
[#]CL-3 is the parent compound from which all of the other synthetic compounds were derived.
[##]SK-03-92 was provided at 1.024 µg/µL (all others 10.24 µg/µL), but was diluted in the same manner as the other compounds in the assay.
[*] Extracts marked with an asterisk consistently showed activity (distress, paralysis, and/or death) at the lowest tested concentrations.

Developmental Assay

Thirteen synthetic compounds were tested in the developmental assay. Six were chosen because they showed activity in the motility assay, while the remaining seven where chosen based on similarities in their molecular structure to those six compounds. Variations in developmental progress were noted by comparing worms in the treated wells to worms in the wells containing only M9 buffer (Table 4). The majority of worms treated with the lowest concentrations of the synthetic compounds developed at the same rate as worms in the untreated wells (-) and the two highest concentrations were lethal (+++) to the worms for twelve of the sixteen synthetic compounds. Some compounds caused severe distress (++) to nematodes in the test wells. There were five instances of worms that were delayed, but otherwise healthy (+). Three compounds (CL-5, SK-05-22, and VR-072309-01) showed death or delays in development at the lowest two concentrations.

Table 4. Developmental assay data after four days.

Synthetic Compound	1/1600	1/800	1/400	1/200	1/100
M9 Only	Adults, many L1s and embryos	Adults, many L1s and embryos	Adults, many L1s and embryos	Adults, many L1s and embryos	Adults, many L1s and embryos
A6	-	-	+	+++	+++
A8	-	-	-	+++	+++
A9	-	-	+++	+++	+++
A10	-	-	++	+++	+++
CL-3	-	-	-	+	+++
CL-4	-	-	+++	+++	+++
CL-5*	+	+++	+++	+++	+++
SK-03-28F2	-	-	-	+++	+++
SK-04-03	-	-	-	+	+++
SK-04-08	-	-	-	+++	+++
SK-04-23	-	-	+	+++	+++
SK-04-50	-	-	-	+	+++
SK-05-16	-	-	-	-	++
SK-05-17	-	-	++	++	+++
SK-05-22*	-	+	+++	+++	+++
VR-072309-01*	-	+++	+++	+++	+++
Ive	++	++	+++	+++	+++
M9 + DMSO	-	-	-	-	-

[1] Worms were scored as dead (+++), distressed (++), slightly delayed (+), or the same as the M9 only untreated wells (-).
[2] Serial dilutions of DMSO in M9 (highest DMSO concentration was 1%) and ivermectin (highest ivermectin concentration was 1ng/µL) were also included.
[*] Extracts marked with an asterisk consistently showed activity (distress, paralysis, and/or death) at the lowest tested concentrations.

DISCUSSION

With the recent increase in the incidence of resistance to many of the currently available anthelmintics, new drugs are desperately needed. Naturally derived products, in the form of crude fungal extracts and a series of synthetic compounds, were investigated for anthelmintic properties in this study.

As part of this project, two new methods were devised for screening natural compounds for anthelmintic activities using low volumes of extract. Previous studies attempting to identify anthelmintic drugs required larger quantities of the compounds to be tested (Kaminsky et al., 2008). Large amounts of compounds are not always obtainable for study due to the availability of the compound or the cost associated with collecting them. By developing assays that use only a few micrograms or nanograms as was the case with the synthetic compounds, of each extract makes it possible for researchers to do more with less compound and increase the amount of resources available to focus on identifying the active molecule(s) and mechanism of action for any extracts identified as active.

Six fungal extracts were identified in the motility assays that caused paralysis or death in *C. elegans* (Table 2). When more of these fungi can be obtained, it may be possible to identify the active molecule(s) responsible for causing anthelmintic activity. Researchers will need to separate the extracts into fractions and retest each one on *C. elegans*. After isolating the active fraction(s), other experiments can be performed to identify the specific molecule within each fraction that is causing the activity in the test organism. By identifying the fungal molecule that causes the paralysis in the nematodes, it can be determined whether or not the molecule has been identified previously.

For the six synthetic compounds that showed activity against the worms in the motility assay (Table 3), the active molecule is already known. This study used concentrations of the compounds that were between 6 to 100-fold higher than the concentration of ivermectin that showed activity in our work. In order for the compound to be pursued as a potential drug, the lowest concentration required for activity would need to be determined. Chemists could also work to modify the active compounds, changing their functional groups and retesting them to see if the modifications increase the potency of the compound.

The developmental assay, although less successful at identifying active compounds than the motility assay, still created a starting point for further research into anthelmintics that cause developmental arrest or prevent the production of viable offspring in the worms. An assay of this kind was also used in the recent study that identified amino-acetonitrile derivatives (AADs) as new anthelmintic agents with market potential (Kaminsky et al., 2008). Since most of the drugs currently available work by causing paralysis and death in the helminths (Table 1), specifically looking for compounds that cause developmental arrest or decreased fecundity within the parasites has the most potential for new targets to which drug resistant nematodes have not already developed resistance. Three compounds were identified that seemed to slow the development of the *C. elegans* (Table 4), but most still resulted in viable embryos. Future studies could look into the remaining synthetic derivatives to see if any of them would be more effective than the thirteen screened for this study. Some modifications would need to be added to this assay for the screening of the fungal extracts, which require higher concentrations of the extract. Increasing the amount of fungal extract will

expose the L1 stage worms to concentrations of DMSO over 1%. Concentrations at that level are lethal to the L1 stage worms used in the study. Many of the fungal extracts also have large amounts of debris which make visualizing the worms very difficult. The developmental assay involves keeping the worms exposed to the extracts for several days, so new ways of filtering out fruiting body debris will be needed to ensure the worms are allowed to stay in their test wells for the duration of the assay.

In order for new compounds with anthelmintic activity to be useful, they need to be potent, but also function through a novel molecular mechanism. One future avenue of research would be to test the synthetic derivatives that showed activity in the motility and developmental assays on mutant strains of *C. elegans* that are resistant to commonly used anthelmintics, like ivermectin. These strains possess genetic changes that make them less susceptible to the molecular pathways utilized by established anthelmintic drugs. Any compound that impacts the drug resistant nematode's ability to function would be using a different molecular pathway. The two assays would be performed as described here, but mutant worms could be tested in addition to N2/wild-type worms. If any compounds showed activity against the resistant strains, the power of *C. elegans* as a genetic model system could be exploited to identify the mechanism of action for the new compound, as has been done for existing anthelmintics (Holden-Dye & Walker, 2007). Specifically, N2 worms would be exposed to a chemical mutagen, like ethylmethane sulfonate, and the hermaphrodite progeny of those animals (F1 generation) would be singled onto culture plates that are laced with the compound of interest. The F2 generation would develop on the culture plates with the drug, and if they are sensitive to the drug, they would either die by developmental arrest or muscle paralysis resulting in starvation. If the F2 progeny

have a molecular lesion in a gene whose protein product is required for the animals to respond to the drug, then the progeny would develop normally on the plate. The mutated gene in these animals could be identified by molecular mapping techniques and a more detailed analysis would follow.

Because the incidence of drug resistant nematode parasites is steadily increasing, it is important to have a means of identifying compounds to fight them. Naturally derived compounds, like the stilbenes, are receiving attention for their many benefits to model organisms and the plants from which they were derived. Although this study only identified a few active compounds, it can serve as a starting point for future research. The two assays developed in this study will be useful tools in the quest to identify new anthelmintic compounds. These assays have the power to identify anthelmintic activity using small quantities of test compounds, saving money and resources for researchers.

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