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Evaluation of Anthelmintic Properties and Toxicity of Pure Compounds Isolated from Dalea Spp (Plantae, Fabaceae)

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EVALUATION OF ANTHELMINTIC PROPERTIES AND TOXICITY OF PURE COMPOUNDS ISOLATED FROM *DALEA* SPP (PLANTAE, FABACEAE).

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ABSTRACT

EVALUATION OF ANTHELMINTIC PROPERTIES AND TOXICITY OF PURE COMPOUNDS ISOLATED FROM *DALEA* SPP (PLANTAE, FABACEAE).

by

Victoria LH McPherson

November 26, 2018

Infecting upwards of a billion people worldwide, hookworm is one of the most prevalent parasitic infections affecting the world today. Those infected experience hookworm disease, characterized by severe anemia, which can lead to malnutrition, low birth weights, and physical and mental impairments. Currently, the benzimidazoles albendazole and mebendazole are used as the primary treatment; however, resistance to these drugs is emerging. Due to this resistance, finding new anthelmintic compounds active against hookworm is a priority. Previously, a pure compound isolated from *Dalea ornata* was identified as having strong anthelmintic activity when used against the hookworm *Ancylostoma ceylanicum*. This project was continued using pure compounds isolated from *Dalea pogonathera* and *D. parryi*. The pure compounds were tested for toxicity and anthelmintic properties. The structure of these compounds were determined using NMR. To test toxicity, spleen cells were obtained from Golden Syrian hamsters, *Mesocricetus auratus,* which were then exposed to 50µg/mL of compound for 24 hours to determine whether the compound induces apoptosis. This was done by analyzing the cells after the 24 hour

incubation using flow cytometry to compare cell survival from the experimental cells to the controls. An *ex vivo* assay was employed to test for anthelmintic properties by exposing adult *A. ceylanicum* to 50µg/mL of compound for 5 days, monitoring motility and mortality. Unfortunately, none of the pure compounds tested were active towards *A. ceylanicum*; however, the methods used can be replicated for similar projects.

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INTRODUCTION

Hookworm Epidemiology

Hookworm is one of the most prevalent parasitic infections in the world today. Infecting an estimated billion people worldwide, hookworm is an endemic infection in Africa, China, Southern Asia, and South America, with 240 million people being infected in Sub-Saharan Africa alone (Hotez et al. 2004; Brooker et al. 2010). Furthermore, an estimated 5.08 billion people are at risk, with 1 billion of those being school-aged children (Pullan and Brooker 2012). These infections are responsible for around 4 million disability adjusted life years (DALYs) and an economic loss of up to $139 billion dollars (Bartsch et al. 2016). The hookworm species responsible for causing these infections in humans are *Necator americanus* and *Ancylostoma duodenale*, with occurrences of a zoonotic infection of *Ancylostoma ceylanicum* (de Silva et al. 2003; Hotez et al. 2004; Brooker et al. 2010; Ngui et al. 2012). Depending on the worm burden, specifically the number of adult worms residing in the small intestine, the infection can cause hookworm disease, which is characterized by its primary symptom of iron-deficiency anemia (Guyatt et al. 2000; Crompton and Nesheim 2002; Humphries et al. 2011). This anemia is caused by hookworms feeding on blood while it resides in the host's small intestine. The adult hookworm latches on to the endothelial cells of the small intestine via its buccal capsule and creates negative pressure to feed off of the flow of blood and plasma, producing anticoagulant secretions that inhibit the function of platelets

(Cappello et al. 1996, Chadderdon and Cappello 1999, Del Valle et al. 2003). If the amount of blood lost is greater than that which is being produced, the end result is anemia and hypoproteinemia, an abnormally low level of protein in the blood (Crompton and Nesheim 2002).

The most at risk for this infection are those living in poverty with minimal sanitation (Hotez 2008; Humphries et al. 2011). This disease is spread rapidly through communities because it is primarily transmitted through contact with soil contaminated by feces, although, for *Ancylostoma* species an oral route of transmission can occur (Ngui et al. 2012). Although all people living in poverty in an endemic area are at risk for the disease, children and pregnant women are the most at risk for complications associated with hookworm disease. Iron-deficiency anemia and hypoproteinemia are associated with stunting growth both physically and mentally in children (Sakti et al. 1999; Guyatt et al. 2000), malnutrition (Crompton and Nesheim 2002; Hotez et al. 2004), and pregnancy complications such as low birth weight and impairment of milk production (Santiso 1997). Although mortality rates for hookworm infection are low, there have been a few studies suggesting chronic hookworm infection increases the susceptibility of the host to other infections such as malaria (Mwangi et al. 2006; Humphries et al. 2011), tuberculosis (Potian et al. 2011), and HIV (Borkow et al. 2007; Walson and John-Stewart 2007).

Hookworm Life Cycle

While residing in the small intestine, female hookworms produce approximately 30,000 eggs per day, varying with species, which then leave the body in feces. The eggs hatch, the larvae molt twice, and then become infective during their third stage (L3). At this stage they produce proteases allowing them to penetrate the skin (Loukas et al. 2005), most commonly being the palms of hands and the soles of feet, get into the blood stream, and make their way to the lungs. While in the lungs, the L3 are coughed up into the trachea, swallowed, and then arrive at the small intestine. This is where the larvae mature into adult hookworms, feed, release eggs, and begin the cycle once more (Figure 1). During the act of feeding, adult hookworms continue to be motile in the small intestine, moving every 4-6 hours after feeding at a single spot, resulting in a blood loss of about 0.02-0.2 mL of blood per worm per day (Sakti et al. 1999). This loss of blood can result in the primary symptom of hookworm disease, iron-deficiency anemia.

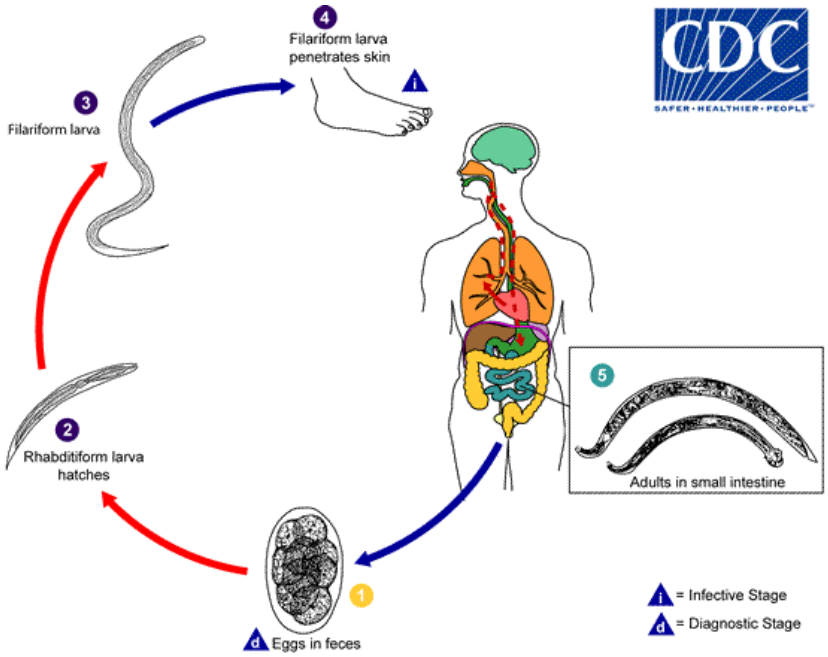

Figure 1.1. The Life Cycle of Hookworm.
Depicted is the life cycle of hookworm, starting at the egg released in the feces (1) until the maturation from larva to adult worm in the small intestine (CDC 2013). Not pictured is the second route of infection, oral transmission.

Hookworm Immunology

As with most pathogens, hookworm infection elicits an immune response. To clear a hookworm infection, it is necessary for the adaptive immune response to be activated, which involves the maturation of CD4+ T lymphocytes. Specifically, a T helper 2 (TH_2) response is required. A TH_2 response is one of the subsets of the adaptive immune response characterized by the activation and differentiation of B cells into plasma cells. Plasma cells have the ability to produce and secrete antigen specific antibodies, proteins that can bind to antigens and activate other cells of immunity. To successfully clear a hookworm infection, IgE must be produced and basophils, eosinophils, and mast cells need to be activated (Quinnell et al. 2005;

Loukas et al. 2005; McSorley and Loukas 2010; Urb and Sheppard 2012). In response to a hookworm infection, interleukin-4 (IL-4) is secreted by basophils that have either acquired antigen or in the presence of dendritic cells acting as antigen presenting cells, initiating a TH_2 response by causing the differentiation of naïve T cells to TH_2 cells while also preventing a TH_1 dominant response (Gaze et al. 2012; Yoshimoto 2018). A TH_1 response is not necessary and would not aid in hookworm clearing because it involves the activation of macrophages, phagocytic cells, that are unable to engulf hookworm due to their large size. The activated TH_2 cells then secrete IL-5 and IL-13, which activate eosinophils and facilitates the secretion of IgE respectively (Gaze et al. 2012). Secreted IgE will then bind to the hookworm antigen using the variable region of its structure, leaving the constant region (Fc) to be bound by eosinophils or mast cells. Once bound, eosinophils and mast cells will release cytokines and reactive oxygen species that are toxic to the hookworm and eventually result in death. This immune response generally occurs in the blood, during the larval hookworm's migration. Once in the small intestine, the antibody IgA contributes to the mucosal immune response. Involved with protecting mucosal surfaces, such as the small intestine, IgA is potentially responsible for the neutralization of hookworm secretions, initiation of eosinophil degranulation, and can interfere with hookworm feeding (Bungiro et al. 2008; Moreau and Chauvin 2010).

However, hookworm does have methods to evade the immune system. One of the first evasion tactics employed is antigenic shedding. It is believed that

hookworm larva is covered in a larval sheath that they cast once they have infected a host to distract the immune system (Kumar and Pritchard 1992). Instead of preventing an immune response, it uses the distraction of an immune response to complete the migration to the small intestine. Although the mechanism is not known, it has also been shown that hookworm reduce dendritic cell differentiation and suppresses the proliferation of antigen presenting cells in general (Dondji et al. 2008; Passos et al. 2017). As mentioned previously, dendritic cells are integral to the activation of the adaptive immune system and, by preventing their differentiation, the immune response is slowed. Hookworm also has been shown to suppress and induce apoptosis in CD4+ T cells (Chow 2000; Dondji et al. 2010; Morawski et al. 2017). Another way hookworm disrupts the TH_2 response is to induce Natural Killer cells (NK cells). This induction allows NK cells to produce interferon-γ, which is a cytokine involved with the upregulation and maintenance of a TH_1 response (Bethony et al. 2006). Besides preventing a TH_2 response from occurring, hookworm can also reduce the effect of a TH_2 response. One of the mechanisms behind this is the production of proteases. Hookworms have been shown to produce proteases that cleave the Fc region of antibodies (Pritchard 1995). This means that the antibodies can bind to the antigen, but not allow the activation of eosinophils or mast cells, thereby saturating the antibody binding regions with ineffective antibodies. Hookworm can also produce metalloproteases, which cleave eotaxin, a protein that recruits eosinophils to the site of infection (Culley et al. 2000). Along with proteases, hookworm also release excretory/secretory (ES) proteins. These

proteins have been shown to induce a hookworm-like immune response, even when hookworm is not present. They upregulate nitric oxide production, which impacts lymphocyte proliferation, IL-12, and interferon-γ, while decreasing IgG and IL-4, impairing the TH_2 response (Dondji et al. 2008; Diliani and Dondji 2017). ES is also hypothesized to induce a specific dendritic cell phenotype, one that is tolerogenic (Logan et al. 2018). Tolerogenic dendritic cells, specifically CD103+ dendritic cells, are responsible for maintaining homeostasis in the intestinal mucosa. They are induced by the production of IL-10 from TH_2 T cells and activate FoxP3+ T regulatory cells (T_{reg}). The primary function of tolerogenic dendritic cells and T_{reg} cells is to modulate the immune system by immunosuppression. Essentially, they produce anti-inflammatory cytokines like those of IL-10 and TGF-β that prevent the differentiation and proliferation of naïve T cells into effector T cells (Ricci et al. 2011). Related to this is the activation and polarization of M2 macrophages. Damaged epithelial cells release IL-33, which propagates T_{reg} and M2 macrophage differentiation, to begin immunosuppression and a healing response (Gordon and Martinez 2010; Takenaka and Quintana 2017; Schwartz et al. 2018). By activating cells of immunity involved in suppression, hookworm prevent an immune response by invoking self-tolerance. This mechanism of enforcing self-tolerance is one of the reasons those chronically infected with hookworm do not have allergies. This effect is also amplified in the lungs during larval migration, which is hypothesized to be the mechanism responsible for the decrease in asthma in those infected (Schwartz et al. 2018). Lastly, as previously mentioned, hookworm needs a steady flow of blood to

feed. To do this, they produce anticoagulants to inhibit and prolong the period of time it takes for blood to clot (Schuster et al. 2013).

Current Treatment Methods

Current treatment of hookworm infection is completely reliant on the use of benzimidazole anthelmintics, specifically mebendazole (MBZ) or albendazole (ABZ), distributed following the deworming campaigns endorsed by the World Bank and World Health Organization (WHO 2001; World Bank 2003). These campaigns call for mass drug administration to high-risk groups (school-aged children). Although cure rates following single dose regiments previously were recorded as high as 100%, newer studies suggest this number is much lower, with a failure rate of 39-56% (Humphries et al. 2011; Humphries et al. 2013). A recent study conducted by Palmeirim et al. compared a single dose treatment of 500mg to three doses of 100mg mebendazole against soil-transmitted helminths. The cure rate of the single dose treatment was drastically lower, 13%, compared to the 97.9% cure rate of the multiple dose treatment (Palmeirim et al. 2018). Unfortunately hookworm was not present as one of the soil-transmitted helminths included in this clinical trial. However, in a study including hookworm, the cure rate was 25.5% for a single dose of 500mg mebendazole (Mrus et al. 2018). With the need to use multiple doses, concerns have been raised about the selective pressure put on hookworm and potential resistance arising from it. This is significant especially given the high failure rates of recent field studies, suggesting resistance may be emerging (Furtado et al.

2016).

Using MBZ and ABZ may treat the infection, but they do not provide lasting immunity to hookworm infection. This means that even if the treatment is successful, the rate of re-infection still remains high (Albonico et al. 1995). This is why an attempt at a hookworm vaccine occurred, to decrease the rate of re-infection (Hotez et al. 2013). However, despite the high hopes for the success of the trial, the vaccine trial was halted due to the allergic reactions occurring in those previously exposed to hookworm after administration. The same research group has recently modified the vaccine by changing the target. Instead of using a larval protein, the vaccine target is now an adult hookworm protein belonging to *N. americanus*, Glutathione – S – Transferase (Na-GST-1) (Diemert et al. 2017). It is important to note that the vaccine target does differ from MBZ and ABZ's target. The benzimidazoles prevent the formation of microtubules which prevents or minimizes the uptake of glucose. Without glucose, the hookworm is unable to produce ATP and eventually dies. The Glutathione – S – Transferase on the other hand targets the feeding behavior of hookworm by preventing the detoxification of reactive oxygen species. Hookworms digest blood by breaking it down into smaller molecules such as heme and hematin. Both of these molecules can generate reactive oxygen species which are toxic to the hookworm (Diemert et al. 2017). If successful, this vaccine would treat hookworm rather than prevent larval migration. Another vaccine in development is a cysteine protease inhibitor K11777, which has shown a promising effect in increasing the mortality of larvae and adult *A.*

ceylanicum hookworms (Vermeire et al. 2016).

Anthelmintic and Antiparasitic Natural Products

Using natural products to treat infection and disease is not a novel concept. There is evidence that natural products have been used in ancient civilizations such as the Egyptians and Mesopotamians, with the earliest written record of plant-based therapies dating back to 2600 BCE (Dias et al. 2012). Currently, around 80% of the world's population is reliant on natural products for their primary healthcare (Ekor 2013). Along with general malaise, plant compounds have also been used to treat parasitic infections. Artemotil, a derivative of artemisin, is a compound isolated from *Artemisia annua* that has anti-malarial properties (Dias et al. 2012). Also belonging to the *Artemisia* family, *Artemisia parviflora* and *Artemisia siversiana* have anthelmintic properties active against the nematode *Haemonchus contortus*. At 12.5mg/mL, the *A. parviflora* extract killed over 60% of adult worms and over 50% of L3 larva. At 25mg/mL, the *A. siversiana* extract killed over 60% of adult worms and L3 larva (Irum et al. 2017). Another compound found to have anthelmintic properties is in *Eucalyptus globulus,* which has activity against nematodes in goats. Using a 7.5mg/kg dosage, a 66% reduction in the fecal egg count was recorded 21 days post-treatment (Kanojiya et al. 2015). Lastly, an extract from *Combretum mucronatum* was active against *Caenorhabditis elegans*, a non-pathogenic nematode, causing a mortality rate of 58% at 1mg/mL (Agyare et al. 2014). *Dalea spp.* are plants of interest due to their already documented medicinal properties,

such as *D. versicolor*'s ability to enhance the activity of antibiotics against resistant bacterium (Belofsky et al. 2004) and *D. elegans*' antioxidant properties (Elingold et al. 2008). A recent study published from the Dondji and Belofksy labs at Central Washington University indicated that tephrosin, a *Dalea ornata* isolate, had anthelmintic properties against the hookworm *Ancylostoma ceylanicum* (Table 1). After exposing adult worms to 25µg/mL of tephrosin (compound 10), all were dead after 72 hours (Deardorff et al. 2016). However, tephrosin is also highly toxic, making it unusable as a therapy for now. To continue this project, compounds isolated from *Dalea parryi* and *Dalea pogonathera* were evaluated for anthelmintic properties and toxicity.

Table 1.1. Ex Vivo Anthelmintic Activity of *D. ornata* Compounds **1-10** Toward *A. ceylanicum*.
With a concentration of 25µg/mL, compound 10, tephrosin, had killed 97% of hookworms after 48 hours. By 72 hours, all of the worms were dead (Deardorff et al. 2016). Through this experiment, Tephrosin exhibited anthelmintic properties and was very active against *A. ceylanicum*.

	% survival of *A. ceylanicum* relative to control[b] at 25 µg/mL					
compound	day 1	day 2	day 3	day 4	day 5	corresponding µM concentrations
1	100	97 (±3.3)	90 (±0.0)	83 (±5.7)	83 (±5.7)	7.3
2	100	100	100	100	100	8.6
3	100	93 (±3.3)	83 (±5.7)	80 (±5.7)	73 (±3.3)	8.2
4	100	100	100	100	93 (±6.7)	6.1
5	100	100	90 (±5.7)	80 (±5.7)	80 (±5.8)	7.3
6	100	97 (±3.3)	93 (±3.3)	93 (±3.3)	87 (±3.3)	7.0
7	100	100	100	97 (±3.3)	90 (±5.8)	6.1
8	100	100	100	100	100	5.8
9	100	50 (±5.8)	40 (±0.0)	0	0	6.3
10	77 (±5.8)	3 (±3.3)	0	0	0	6.0

CHAPTER 2

THE EVALUATION OF TOXICITY AND ANTHELMINTIC PROPERTIES OF PURE COMPOUNDS ISOLATED FROM *DALEA POGONATHERA*

ABSTRACT

Infecting around 1 billion people, hookworm is one of the most prevalent parasitic infections. Chronic infection can lead to severe anemia, malnutrition, and increased susceptibility to other infections. The current treatments used are the benzimidazole derivatives, mebendazole and albendazole. Although previously successful, emerging resistance has been reported and the need to identify new anthelmintic compounds is high. Pure compounds were isolated from *Dalea pogonathera* using various techniques such as vacuum liquid and thin layer chromatography. The structure of these compounds were then identified using NMR and used in toxicity assays to determine whether they induced apoptosis in the spleen cells of Golden Syrian hamsters, *Mesocricetus auratus*. Of the 16 compounds tested at 50µg/mL, 7 were non-toxic and the remaining 10 ranged from slightly toxic to toxic. The compounds were also tested in an *ex vivo* assay to determine if anthelmintic properties were present. Monitored for motility and mortality at 50µg/mL for five days, none of the 16 compounds were significantly active against adult *Ancylostoma ceylanicum*.

Being one of the most prevalent parasitic infections to date, hookworm infects roughly a billion people worldwide. It is endemic in Africa, Southern and Southeast Asia, and Central and South America, with an increasing prevalence in areas of rural poverty.[1] The hookworms *Necator americanus, Ancylostoma duodenale*, and *Ancylostoma ceylanicum* are those responsible for the majority of human infections; however, there are some accidental infections most commonly associated with dog hookworms such as *Ancylostoma caninum.*[2-6] When infected, the hosts release feces containing eggs that will hatch in a warm and moist environment, releasing larva. The larva will then undergo two molts, becoming the infective L3 larva. The L3 are able to pierce through the skin, use the bloodstream to travel to the lungs where it causes irritation. This irritation causes the host to cough, bringing the larva into the trachea where it is then swallowed, eventually bringing them to the small intestine where the L3 mature into adult hookworms. As adults, the hookworms latch onto the endothelial cells lining the small intestine using their buccal capsules and feed off the flow of blood and plasma, producing anticoagulant secretions that inhibit the function of platelets.[6-10] During the act of feeding, adult hookworms continue to be motile in the small intestine, moving every 4-6 hours after feeding at a single spot, resulting in a blood loss of about 0.02-0.2 mL of blood per worm per day.[10] Depending on the hookworm burden, this can cause anemia, the primary symptom of hookworm disease.[11] *A. duodenale* and *A. ceylanicum* also have an oral route of transmission, involving the direct ingestion of L3 larva from the environment.

Although all living in endemic areas are at risk for infection, those living in poverty have an increased prevalence of disease.[12,13] The most at risk for complications are pregnant women and children. Iron-deficiency anemia and hypoproteinemia are associated with stunting growth both physically and mentally in children, malnutrition, and pregnancy complications such as low birth weight and impairment of milk production.[1,10,11,14,15] Chronic infection is also associated with reduced lymphocyte proliferation and increased apoptosis of CD4+ T cells and an increase in nitric oxide production.[16-18] More recently, hookworm and other soil-transmitted helminths have been discovered to activate tolergenic dendritic cells, T regulatory cells, and M2 macrophages.[19-23] All of these cell types promote self-tolerance, inhibiting the immune response. This regulation of the immune response may be responsible for the increased susceptibility of those chronically infected to tuberculosis, HIV, and malaria.[13,24-27]

Currently, the primary drugs used to treat hookworm infection are the benzimidazole derivatives, mebendazole (MBZ) and albendazole (ABZ). Following the World Health Organization and World Bank's deworming campaign, the mass drug administration of single-dose MBZ and ABZ were widely distributed to high-risk groups.[28,29] Although cure rates following single dose regiments previously were recorded as high as 100%, newer studies suggest this number is much lower, with a failure rate of 39-56% seen in field studies.[13,30] A recent study conducted by Palmeirim et al. compared a single dose treatment of 500mg to three doses of 100mg mebendazole against soil-transmitted helminths. The cure rate of the single

dose treatment was drastically lower, 13%, compared to the 97.9% cure rate of the multiple dose treatment.[31] Unfortunately hookworm was not present as one of the soil-transmitted helminths included in this clinical trial. However, in a study including hookworm, the cure rate was 25.5% for a single dose of 500mg mebendazole.[32] With the need to use multiple doses, concerns have been raised about the selective pressure put on hookworm and potential resistance arising from it.[33] This is significant especially given the high failure rates of recent field studies, suggesting resistance may be emerging.[13,30,31] New potential candidates with a different mechanism of action than the benzimidazoles are desirable.

Dalea, a plant genus belonging to the Fabaceae family, is native to the New World. *Dalea spp.* have been previously shown to have medicinal purposes such as increasing the activity of antibiotics and antioxidant properties.[35,36] Tephrosin, a pure compound isolated from *Dalea ornata,* is very active against adult *A. ceylanicum*, causing the mortality of all of the worms by day three of an *ex vivo* trial.[34] However, tephrosin is also toxic, making it unusable as a therapy for now. Due to the identification of an active compound, *Dalea spp.* are still of interest. To continue this work, *Dalea pogonathera,* native to Arizona, New Mexico, and Texas, has been evaluated for the presence of anthelmintic isolates.

Results and Discussion

After the *D. pogonathera* extracts were purified via linear solvent gradient chromatography (Figure 1), two bioassays were completed to determine the toxicity

against splenocytes acquired from Golden Syrian hamsters, *Mesocricetus auratus*, and evaluate the anthelmintic properties towards *A. ceylanicum*.

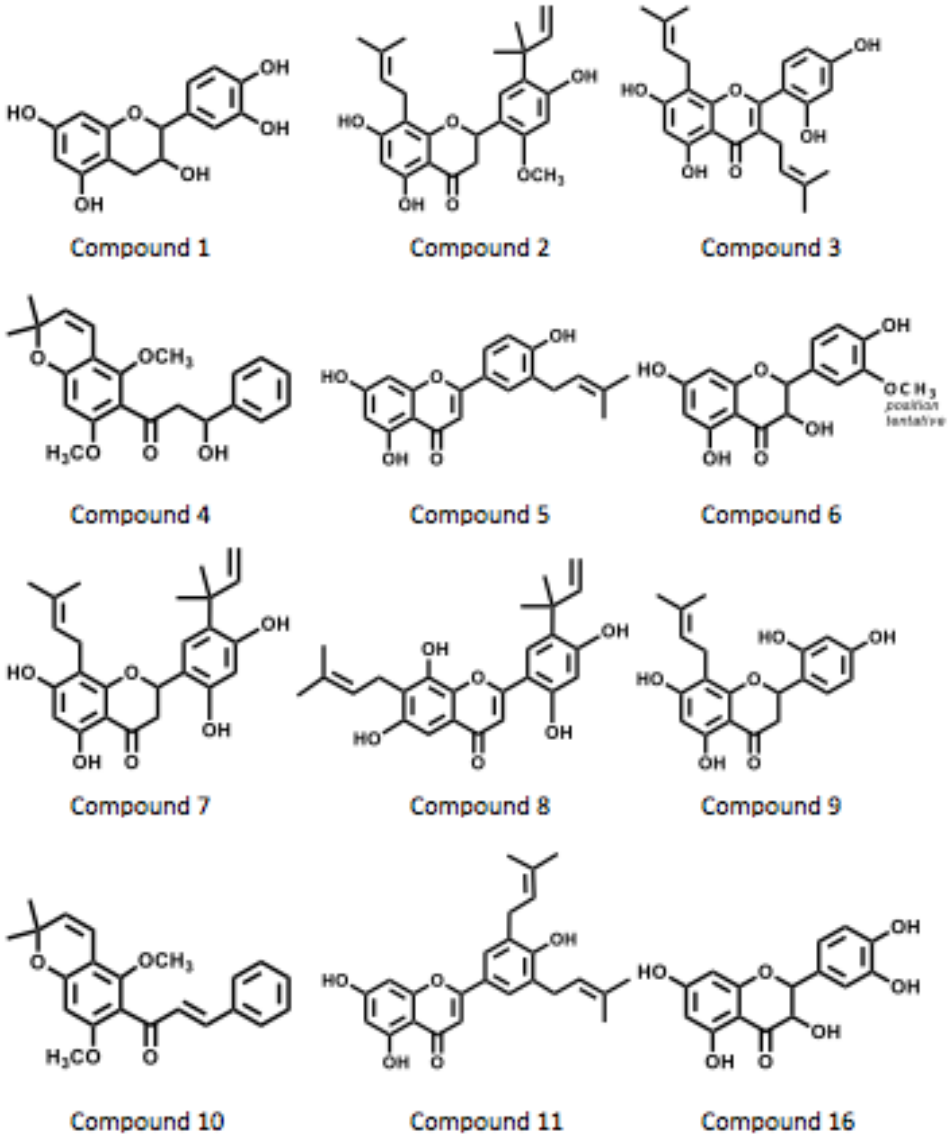

Figure 2.1. Structures of *D. pogonathera* Compounds **1-11, 16**

Table 2.1. Toxicity of *D. pogonathera* Compounds **1-16** at 50µg/mL Relative to No Treatment Control

compound	% compound live cells	% control live cells	toxicity
1	47.2	33.1	Non-toxic*
2	0.31	40.5	Toxic*
3	0.31	40.5	Toxic*
4	7.65	29.8	Toxic*
5	24.7	38.6	Toxic*
6	34.8	34.1	Non-toxic
7	0.66	34.9	Toxic*
8	0.13	34.9	Toxic*
9	9.3	36.5	Toxic*
10	0.4	36.5	Toxic*
11	0.53	36.5	Toxic*
12	0.17	36.5	Toxic*
13	44.9	52.2	Non-toxic
14	51	52.2	Non-toxic
15	55.5	52.2	Non-toxic
16	77.2	66.9	Non-toxic*

*Statistically significant difference

Comparing the percentages of live cells of 10^6 cells of the 50µg/mL treated wells to that of the no treatment wells containing 2% DMSO using flow cytometry, the toxicity of each compound was determined (Table 1). The toxicity, for the context of this paper, is defined as the percentage of live cells in the experimental wells being statistically significantly lower than the percentage of live cells from the control wells. Compounds 1, 6, and 13-16 were all non-toxic, with compounds 1 and 16 actually performing significantly better than the control, acting potentially as mitogens. The reason for this increased percentage of live cells was not investigated; however, all of the compounds that were non-toxic are promising potential

therapies. The remaining compounds were found to be toxic, with some, like compounds 2 and 12, devastatingly so, with a percentage of live cells of 0.31% and 0.17% respectively.

All of the compounds were then tested using an *ex vivo* assay to evaluate the anthelmintic properties the compounds had towards adult *A. ceylanicum* (Table 2, Figure 1). Unfortunately, none of the compounds exhibited any significant anthelmintic activity. Compound 4 had the most activity with a mean mortality rate of 87.5%, followed by compound 8 with 91.7% (±7.2).

Table 2.2. Ex Vivo Anthelmintic Activity of *D. pogonathera* Compounds **1-16** toward *A. ceylanicum*

	% survival of *A. ceylanicum* relative to control at 50µg/mL				
compound	day 1	day 2	day 3	day 4	day 5
1	100	100	100	100	100
2	100	100	100	100	100
3	100	100	100	100	100
4	100	100	100	100	87.5 (±0)
5	100	100	100	100	91.7 (±14.4)
6	100	100	100	100	100
7	100	100	100	95.8 (±7.2)	91.7 (±7.2)
8	100	100	100	100	100
9	100	100	100	100	100
10	100	100	100	100	100
11	100	100	100	100	100
12	100	100	100	100	95.8 (±7.2)
13	100	100	100	100	95.8 (±7.2)
14	100	100	100	100	100
15	100	100	100	100	95.8 (±7.2)
16	100	100	100	100	100

[a]Data in the table are in mean values ± standard error.

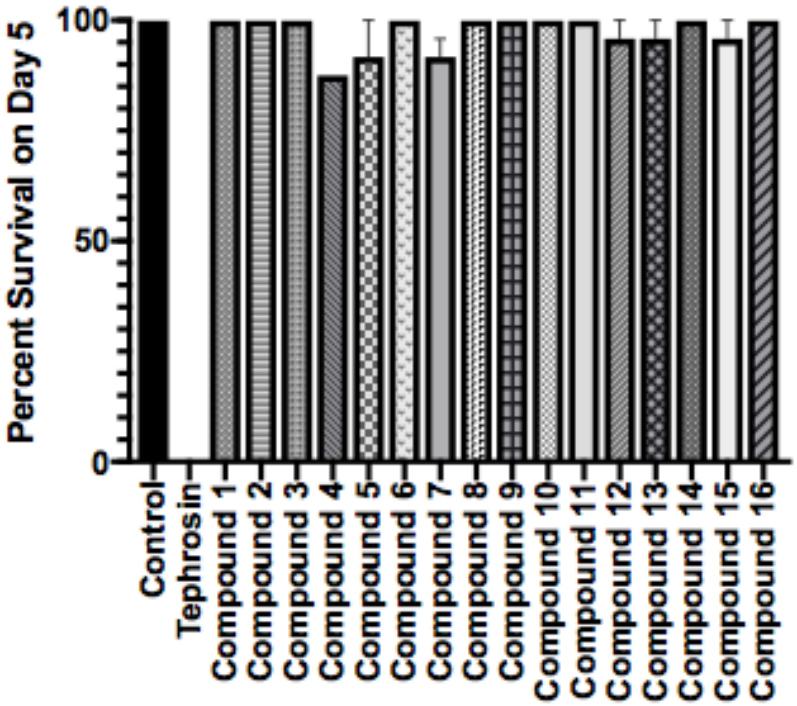

Figure 2.2. Percent Survival of Hookworm on Day 5 of *Ex Vivo*.

Although none of the compounds isolated from *D. pogonathera* were active toward *A. ceylanicum*, the methods used are applicable for similar research involving the screening of compounds. Aside from the biological components, compounds 4 and 8 are novel compounds that have not been structurally identified or added to databases before. It would be interesting to investigate whether these compounds have other medicinal properties not tested in the scope of this paper despite their toxicity.

Being considered one of the World Health Organization's neglected tropical diseases, the need to identify new compounds active toward hookworm is great and more research in developing treatments is necessary, such as the work toward a hookworm vaccine.[37] Not only is this work relevant to areas of high prevalence, but

also to the recently reported reemergence of hookworm in the Southern United States.[38] Hookworm effects over a billion people at a given time, identifying a new active compound is just one step toward eradicating hookworm disease.

Experimental Section

Plant Material. Whole plants of *D. pogonathera* were collected by G. Belofsky in Southeast Arizona.

Extraction and Isolation. The aerial portions of *D. pogonathera* were extracted using methanol, blended, filtered, and the filtrates were evaporated. The crude extract was then fractionated using vacuum liquid chromatography and Sephadex LH-20 chromatography. The fractions created were pooled using thin layer chromatography. To separate these fractions into pure compounds, linear solvent gradient chromatography was used. ^{13}C NMR spectroscopy was used to determine structure and confirm that each compound obtained was unique.

Animals. All animals used in the following experiments were Golden Syrian hamsters (*Mesocricetus auratus*) housed at Central Washington University (CWU), with some being obtained from Simonsen Laboratories (Gilroy, CA, USA). The experiments conducted were approved by the CWU Institutional Animal Care and Use Committee (Protocol Number A021706).

Toxicity Assay. The spleens of euthanized Golden Syrian hamsters were collected and cells were obtained using cell strainers. The cell preparation was then deprived of red blood cells using lysis buffer and spun in the centrifuge for 10mins, 1200RPM

at 4°C. The cells were washed in RPMI-5 [5% heat inactivated fetal calf serum, 1% penstrep (100µg/mL penicillin/streptomycin), 1% L-glutamate (100µg/mL L-glutamate), RPMI-1640] twice, with the cells being spun between each wash. After the washes, the cells were counted and 10^6 cells were plated in RPMI-10 [10% heat inactivated fetal calf serum, 1% penstrep (100µg/mL penicillin/streptomycin), 1% L-glutamate (100µg/mL L-glutamate), RPMI-1640] in each well of a 24-well plate. They were then exposed to a 50µg/mL dose of the pure *Dalea pogonathera* compounds. The positive control used was 5mM menadione, a known toxic compound, and the negative control was 2% DMSO.[39] The experiment was run in triplicate with additional wells for the no-treatment and assay stain controls. The cells were incubated for 24 hours in 37°C, 5% CO_2 and then prepared for flow cytometry analysis. They were stained with Invitrogen's (Carlsbad, CA, USA) annexin V, Alexa Fluor 488 conjugate and propidium iodide and run through the Bio-Rad S3e Cell Sorter, collecting 50,000 events. The data was then compensated to minimize the overlapping of fluorophores, without which can cause the skewing of data due to the overexpression of fluorescence detected by the flow cytometer, and analyzed using FlowJo (Ashland, OR, USA) software. In FlowJo, the data used for further analysis was the percent of cells not dyed. The only cells of concern were those still living and cells dyed by either annexin V or propidium iodide are dead or in the process of dying. Following this, a one-way ANOVA with a post-hoc Tukey comparison was completed to determine toxicity by comparing the percentage of living cells from treated wells to the no-treatment control.

Ex Vivo Assay. 3-6 week old Golden Syrian hamsters were infected with 125 - 150 L3 hookworms. During the 21-day infection, fecal collections were done to collect eggs to maintain the lifecycle of *A. ceylanicum*. After 21 days, the hamsters were euthanized via CO_2 and the adult hookworms were removed from the small intestine and washed as previously described.[34] Following the three 15 minute washes, the worms were incubated for 24 hours at 37°C, 5% CO_2 in RPMI-1640 supplemented with 50% fetal bovine serum, 25µg/mL fungizone, and 20X penicillin/streptomycin. The hookworms were then plated in 24-well plates with 8 - 10 worms per well containing RPMI-1640, 50% fetal bovine serum, 25µg/mL fungizone, 20X penicillin/streptomycin, and 50µg/mL of the *Dalea pogonathera* compound being tested. To avoid bias, one worm was plated per well to each well before adding another, with the pattern of addition changing after each complete cycle. The positive control used was 25µg/mL tephrosin, a *D. ornata* compound that was shown to be highly active against adult hookworm[34], and the negative control was 1% DMSO. The plated worms were incubated at 37°C, 5% CO_2 for 5 days with monitoring of mortality and motility daily. Motility was scored on a scale from 0 to 3, with 3 being normally active and 0 being dead. Percent mortality was calculated by counting the number of dead, 0 motility, worms. The resulting data were analyzed by one-way ANOVA with a post-hoc Tukey comparison to determine if the compound had any significant anthelmintic activity toward *A.ceylanicum*.

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CHAPTER 3

INVESTIGATION OF *DALEA PARRYI* (FABACEAE) METABOLITES FOR ANTHELMINTIC ACTIVITY AGAINST THE HUMAN PATHOGENIC HOOKWORM *ANCYLOSTOMA CEYLANICUM*.

ABSTRACT

Hookworm, a parasite infecting a billion people worldwide causing iron-deficiency anemia, has begun to exhibit resistance to the current treatments used, the benzimidazoles mebendazole and albendazole. Due to this emerging resistance, the identification of new anthelmintic compounds is necessary. Using a combination of vacuum liquid chromatography and Sephadex LH-20 chromatography and other purification techniques, 13 pure compounds were isolated from *Dalea parryi*. The structures of these compounds were determined using HRESIMS, ^{13}C NMR, and DEPT. Of the 13 compounds, only 12 had enough material for biological testing. The 12 compounds were then tested for their toxicity towards lymphocytes obtained from Golden Syrian hamsters, *Mesocricetus auratus*. Using flow cytometry, the percentage of living cells from cells treated with 50µg/mL of compounds for 24 hours was compared to cells with no treatment. Of the 12 compounds tested, 4 were non-toxic, with the remaining 8 being significantly toxic. An *ex vivo* assay was also conducted to determine the presence of anthelmintic properties. Adult hookworms were exposed to 50µg/mL of compounds for 5 days, with motility and mortality being monitored daily. Unfortunately, none of the compounds were significantly active.

Infecting around 1 billion people worldwide, hookworm is a parasitic worm endemic to Africa, Central and South America, and Southern and Southeast Asia causing iron-deficiency anemia.[1,2] Although generally not fatal, chronic hookworm infection has been linked to an increased susceptibility to malaria, tuberculosis, and HIV, malnutrition, and physical and developmental stunting in children.[1,2,3,4]

Infection with hookworm occurs when exposed skin comes into contact with the infective stage larva (L3) located in the soil. L3 pierce the skin and use the host's bloodstream to travel to the lungs. While in the lungs, the larva causes irritation, causing the host to cough and swallow, bringing the larva up the trachea and on to the small intestine. While in the small intestine, the hookworm mature into adults and attach to the small intestine, producing anticoagulants to continuously feed off blood.[3,5-8] Although varying between species, about 0.02-0.2 mL of blood per worm per day is lost.[3] This blood loss is responsible for the primary symptom of hookworm disease, iron-deficiency anemia.

Currently, following the deworming campaigns put forth by World Bank and the World Health Organization, the mass drug administration of the benzimidazoles albendazole and mebendazole are the usual treatment method for hookworm.[10,11] Previously recorded single-dose cure rates were as high as 100%; however, recent field studies refute that, with a cure rate of around 39-56%.[9,12] Multiple dose regiments have more success, with a recent study suggesting a cure rate for soil-transmitted helminths at 97.9%.[13] Due to the increase in dosages, concerns have been raised about the selective pressure put on hookworm and the potential for

emerging resistance.[14] Research in identifying new compounds with anthelmintic properties to eventually replace albendazole and mebendazole is integral to the continued treatment of hookworm disease.

Prior research using *Dalea ornata* was successful in identifying a compound with anthelmintic properties, tephrosin.[15] However, tephrosin is toxic, so it is unable to be used as a therapy for now. To continue this work, pure compounds have been isolated from *D. parryi*, which has never been the subject of chemical investigation, and they have been evaluated for their toxicity and anthelmintic properties.

Results and Discussion

Chemical Investigation. The *D. parryi* crude methanol extracts were tested in an ex vivo bioassay that revealed apparent reductions in survival of *A. ceylanicum* hookworm. At 100 □g/mL, the crude extracts of aerial and root portions exhibited 13% and 10% reductions in survival, respectively, at day 5 of the assay. This level of activity is quite low compared to that of the crude extract of aerial parts of *D. ornata*; >50% reduction in survival on day 2.[15] However, it was expected that activity would increase with further purification, as was the case in that prior study. The crude extracts of *D. parryi* were each fractionated by silica gel vacuum liquid chromatography (VLC) and selected materials from these columns were further separated by Sephadex LH-20 chromatography. Successive stages of purification of compounds over silica gel using linear gradients and step-gradient elution, resulted

in thirteen pure compounds (Figure 1). Of these compounds, 9 of them are new compounds.

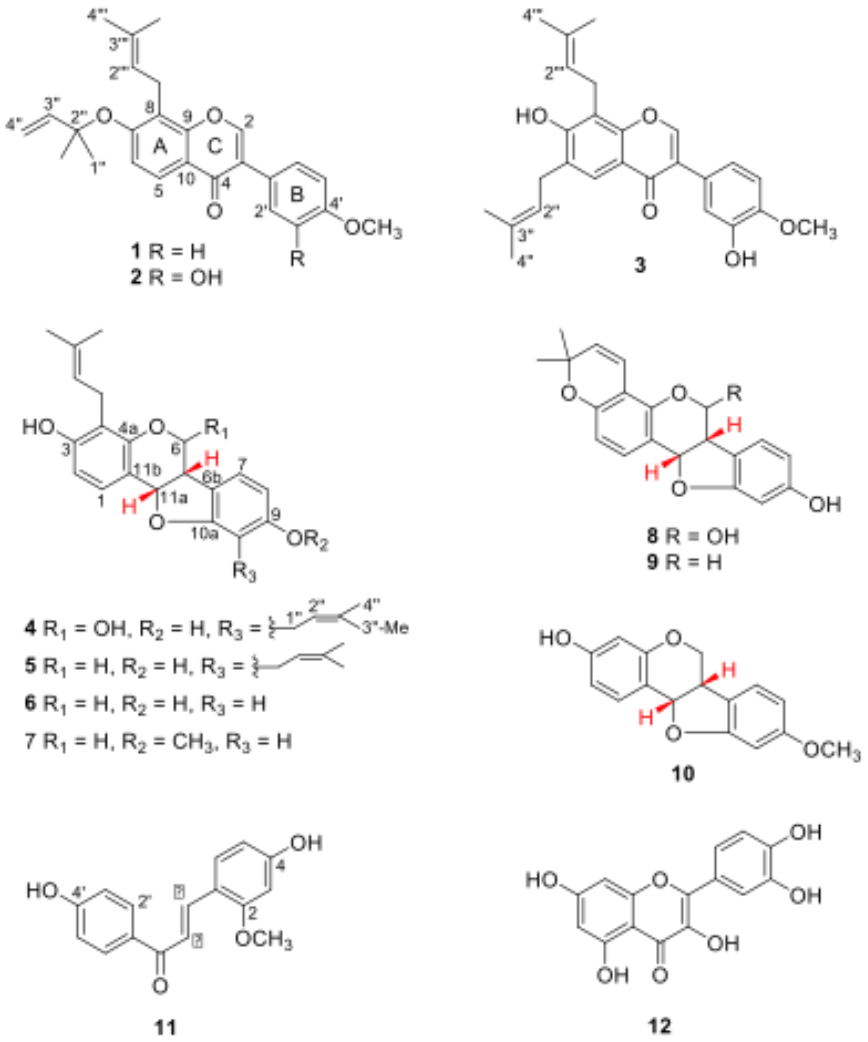

Figure 3.1. Structures of Compounds **1-12** Isolated from *Dalea parryi*.

Biological Investigation. After the 24 hour incubation of cells exposed to 50µg/mL of compound, cell survival was determined using flow cytometry. The percentage of live cells in wells with exposure to compound was compared to the control cells exposed to 2% DMSO and were determined to be toxic if the difference, after a one-way ANOVA with a post-hoc Tukey comparison, was statistically significant. Of the

12 compounds tested, compounds 4, 7, 9, and 13 were found to be non-toxic (Table 1). The remaining compounds were toxic with compounds 5 and 6 having the most destructive effect on the splenocytes. Following the toxicity assay, an *ex vivo* with adult hookworm being exposed to 50µg/mL of compound for 5 days was conducted. The mortality and motility was monitored daily. Unfortunately, none of the 12 compounds tested had any significant anthelmintic properties (Table 1). The lowest percent survival was compound 2 at 83 (± 3.5). Although compounds 5 and 6 were very toxic to mammalian cells, the percent survival of hookworm was 96 (± 5.3) and 100 (± 0.0) respectively, demonstrating that the toxicity toward splenocytes is not indicative of activity toward hookworm.

Table 3.1. Ex Vivo Anthelmintic Activity of *D. parryi* Compounds **1-2, 4-13** toward *A. ceylanicum* Hookworm.

Compound	% Survival of *A. ceylanicum* relative to control[a] after five days of compound exposure at 50 µg/mL	Toxicity expressed as negative or positive effects on splenocyte survival relative to 100% survival (centerline)[b]
1	93 (± 6.7)	-47.6
2	83 (± 3.5)	-86.2
4	88 (± 7.0)	+6.9
5	96 (± 5.3)	-92.5
6	100 (± 0.0)	-90.8
7	88 (± 4.6)	+5.7
8	100 (± 0.0)	-22.8
9	100 (± 0.0)	+45.3
10	100 (± 0.0)	-36.6
11	100 (± 0.0)	-9.4
12	88 (± 7.0)	-21.1
13	88 (± 7.0)	+19.9

[a] Controls of medium plus 1% DMSO all exhibited >95% survival at day five. Data are in mean values ± standard error (SE).
[b] Flow cytometric analyses….+ error ranges.

Although none of the compounds exhibited activity toward hookworm, the chemical investigation and identification of 9 new compounds is relevant in continued hookworm research. The methods used are applicable to similar projects and will hopefully serve its purpose in identifying new compounds to eventually replace albendazole and mebendazole.

Experimental Section

General Experimental Procedures. Melting points were measured on an SRS MPA160 DigiMelt apparatus. Optical rotations were recorded on a PerkinElmer 341 polarimeter (Na lamp, 589 nm); concentrations are reported in g/100 mL. UV spectra

were recorded on an HP-Agilent 8453 photodiode array instrument. ECD spectra were obtained on an Aviv Biomedical, Inc. M400 circular dichroism spectrometer using dry MeOH as the solvent and a 0.1 cm path length quartz cuvette. IR spectra were recorded on a Bruker Alpha spectrometer with universal Alpha T attachment. NMR spectra were obtained on a Bruker Avance 400 MHz system with Topspin 1.3 software. HRESIMS data were recorded on a Waters Q-TOF Premier hybrid mass spectrometer. A Waters Acquity UPLC was used to inject samples in 1:1 MeCN−H_2O using flow injection analysis (100 µL/min) with no intervening column. Preparative linear gradient chromatography employed a custom two-chamber apparatus creating a continuous gradient, with gravity flow (~20 mL/min), over 70-230 mesh silica gel. This was connected to glass columns of varying sizes sealed with PTFE end-fittings. Samples were preadsorbed onto silica gel in solution and evaporated to dryness prior to loading onto the column. Eluting solvent percentages given (see '*Preparation of the extracts and compound purification*') represent estimates, for the sake of reproducibility, of the solvent compositions entering the column. Collection of 20 fractions (20 mL) for example, with a linear gradient of EtOAc (0-100%) in hexanes, will result in fraction 10 having a composition of ~50% EtOAc. Silica gel step-gradient columns also employed 60-100 mesh silica gel, and were run with preadsorbed samples. TLC plates (Sigma-Aldrich; silica gel 60, F_{254}) were eluted with mixtures of MeOH in CH_2Cl_2 or of EtOAc in hexanes, and visualized with UV (254 nm) and the spray reagent vanillin/H_2SO_4 (1 g/100 mL w/v) followed by gentle heating.

Plant Material. Whole plants of *D. parryi* were collected by G. Belofsky, V. Belofsky, and L. Belofsky on April 22, 2015 about two miles up the East End Trail of McDowell Regional Park, Arizona, USA, at GPS coordinates N 33º 40.584', W 111º 48.157', alt. 3404 ft. A voucher specimen was authenticated by Dr. Tom Cottrell, Department of Biological Sciences, Central Washington University, and was deposited in the herbarium of the same department, accession no. 2015005GB. Plant parts were separated, the roots were washed with distilled water and dried briefly, while aerial portions (leaves, stems, and flowers) were air-dried for several days. Dry aerial portions and roots were powdered in a Waring blender and stored in vacuum-sealed plastic bags at -20 ºC prior to extraction.

Preparation of Extracts and Compound Purification. Powdered roots (112 g) and aerial portions (1150 g) of *D. parryi* were extracted separately with 1.5 L and 7 L of MeOH, respectively, in a Waring blender for 2-3 min. The mixtures were each filtered, and the filtrates were evaporated under reduced pressure resulting in 14.9 g of root extract and 135.0 g of aerial portion extract.

The crude root extract was preadsorbed in MeOH solution onto ~10 g of silica gel, the solvent removed under vacuum, and the resulting powder subjected to VLC over a prepacked column bed (10 × 5 cm; i.d. × h) of TLC-grade (230−400 mesh) silica gel. The column was eluted using a stepwise gradient of solvents beginning with hexanes (1 L) and continuing with mixtures (0.75 L each) of EtOAc in hexanes (20, 40, 60, 80, and 100% EtOAc), followed by mixtures of MeOH in CH_2Cl_2 (2, 5, 8, 10, and

30% MeOH). Fractions 3-4 (2.14 g) from this column expressed both the heaviest weight and greatest mixture complexity by TLC analysis. These were further separated by Sephadex LH-20 (Sigma-Aldrich) column chromatography (2.5 × 50 cm) eluting with 1 L of 3:1:1 hexanes-toluene-MeOH, followed by 1 L of 100% MeOH at a flow rate of 0.3-0.5 mL/min with ~8 mL per fraction. The glass column was equipped with a male luer tip for connection to a 100 tube, drop-counting fraction collector. Materials of similar composition as determined by TLC were pooled to give 49 fractions. Compounds **1**-**10** were all isolated upon elaboration of selected subfractions of these Sephadex LH-20 column fractions.

The resulting Sephadex LH-20 fraction 3 (166 mg) was further purified over silica gel (10 × 2.5 cm) using a linear gradient (see '*General Experimental Procedures*') of EtOAc (0-10%) in hexanes. Fractions eluting with ~3-5% EtOAc resulted in **1** (89 mg). The combined Sephadex LH-20 fractions 5-6 (61 mg) were further purified over silica gel (5 × 2.5 cm) using a linear gradient of EtOAc (0-20%) in hexanes. Fractions eluting with 20% EtOAc afforded **2** (23 mg). Combined Sephadex LH-20 fractions 15-21 (45 mg) were further purified over silica gel (9 × 2.5 cm) using a linear gradient of EtOAc (0-30%) in hexanes. Fractions eluting with ~9-12% EtOAc resulted in **9** (8 mg), while later fractions from this column, eluting with 15-30% EtOAc were further purified over silica gel (1.5 × 5 cm) using a step-gradient (see '*General Experimental Procedures*') of EtOAc (0-100%) in hexanes to yield **3** (6 mg) in one fraction, eluting with 30% EtOAc. Fractions 45 and 46 of the Sephadex LH-20 column were combined (51 mg) and chromatographed over silica gel (8 × 2.5 cm) using a linear gradient of

EtOAc (0-50%) in hexanes. Final purification of fractions from this column (14 mg) that eluted with 25-30% EtOAc was achieved over silica gel (1.5 × 6 cm) using isocratic elution with 30% EtOAc in hexanes to provide **4** (7 mg). Fraction 25 from the Sephadex LH-20 column (204 mg) was further purified over silica gel (10 × 2.5 cm) using a linear gradient of EtOAc (0-15%) in hexanes. Fractions eluting with ~8-11% EtOAc resulted in **5** (139 mg). Pooled fractions 38-40 from the Sephadex LH-20 column (41 mg) were further purified over silica gel (7 × 2.5 cm) using a linear gradient of EtOAc (0-25%) in hexanes. Fractions eluting with ~13-20% EtOAc resulted in **6** (11 mg). Fraction 14 from the Sephadex LH-20 column (19 mg) was further purified over silica gel (8 × 1.1 cm) using a linear gradient of EtOAc (0-15%) in hexanes. Fractions eluting with ~1-6% EtOAc afforded **7** (5 mg). Fractions 42-44 of the Sephadex LH-20 column were combined (89 mg) and chromatographed over silica gel (10 × 2.5 cm) using a linear gradient of MeOH (0-6%) in CH_2Cl_2. Materials from this column that eluted with ~2-3% MeOH (75 mg) were chromatographed over silica gel (11 × 2.5 cm) using a linear gradient of EtOAc (0-30%) in hexanes. Fractions eluting with ~23-29% EtOAc (51 mg) were purified over silica gel using a step-gradient of EtOAc (0-30%) in hexanes to yield **8** (38 mg) in fractions eluting with 20-22% EtOAc. Compound **10** (10 mg) was obtained by chromatography of Sephadex column fraction 34 over silica gel (10 × 2.5 cm) using a linear gradient and eluting with ~11-14% EtOAc in hexanes.

The crude extract of the aerial portions (135 g) was preadsorbed in MeOH solution onto ~15 g of silica gel, the solvent removed under vacuum, and the

resulting powder subjected to VLC over a prepacked column bed (10 × 7 cm; i.d. × h) of TLC-grade (230−400 mesh) silica gel. The column was eluted using a stepwise gradient of solvents beginning with hexanes (2 L) and continuing with mixtures (1 L each) of EtOAc in hexanes (20, 40, 60, 80, and 100% EtOAc), followed by mixtures of MeOH in CH_2Cl_2 (2, 5, 8, 10, and 30% MeOH). Fractions of interest 5-7 (2.7 g) from this column were further separated by Sephadex LH-20 column chromatography (2.5 × 75 cm) in an otherwise identical manner to that described above for the root VLC fractions, resulting in 25 fractions. Compound **11** (6 mg) was obtained by chromatography of Sephadex column fraction 14 (64 mg) over silica gel (7 × 2.5 cm) using a linear gradient and eluting with ~42-48% EtOAc in hexanes. A VLC column of Sephadex fraction 23 (85 mg) over silica gel (5 × 3.5 cm) eluting with 100 mL fractions of increasing EtOAc in hexane afforded **12** (16 mg) in a fraction that eluted with 75% EtOAc. Sephadex fractions 15-16 (97 mg) were chromatographed over silica gel (12 × 2.5 cm) using a linear gradient of 0-6% MeOH in CH_2Cl_2. Material that eluted with ~3% MeOH was then purified over silica gel (4.5 × 1.5 cm) using a step-gradient of 0-100% EtOAc in hexanes. Fractions eluting with 60-70% EtOAc consisted of **13** (5 mg).

Anthelmintic and Toxicity Assays. Syrian hamsters (*Mesocricetus auratus)* were used for all animal work including maintenance of the hookworm *Ancylostoma ceylanicum* in the laboratory, ex vivo and toxicity assays. Hamsters were either purchased from Simonsen Lab (Gilroy, CA, USA) or second generation bred at the Central Washington University animal facility. Animals were housed in 12:12 hour

light: dark and temperature-controlled conditions at the Central Washington University (CWU) animal facility. Animal handling and experimental conditions followed protocols approved by the CWU Institutional Animal Care and Use Committee (Protocol Numbers A111608, A111609, and A021706). For each round of the ex vivo assay, 20-25 hamsters were orally infected with 125-150 L3 (infectious stage) hookworm larvae. Initial *A. ceylanicum* larvae were generously provided by Dr. Michael Cappello (Yale School of Medicine, New Haven, CT). On day 21 PI, the fecal material was gathered, the hamsters were euthanized and the small intestines were collected. The fecal material contains hookworm eggs and was used for the production of more infectious larvae for subsequent experiments. Adult worms were harvested from the intestine using tweezers under a dissecting microscope and processed as previously described.[15] Briefly, worms were placed in a Petri dish containing phosphate buffer saline (PBS) with 20 X penicillin/streptomycin (pen/strep), and fungizone (25 µg/mL). The worms were washed three times in RPMI medium containing fungizone (25 µg/mL) and 20X penicillin/streptomycin. After an overnight incubation (37^{o}C; 5% CO_2) in RPMI medium supplemented with fungizone (25 µg/mL) and 20X penicillin/streptomycin, and 50% fetal bovine serum (FBS), active worms were randomly placed in a 24-well tissue culture plate. Each wells contained 8-10 worms. Each compound was tested in triplicate. 1% DMSO was used in negative control wells while tephrosin, a highly active compound isolated earlier from *D. ornata* was used as positive control.[15] All the compounds were tested at 50 µg/mL except the tephrosin positive control that was tested at 25 µg/mL. Plant

materials dissolved in 100% DMSO were diluted in RPMI, fungizone (25 µg/mL), 20X penicillin/streptomycin medium, and 50% FBS medium to reach a final concentration of 1% DMSO. Plates were checked daily for 5 days to determine the percent survival of worms. Dead and living worms were counted to calculate percent survival.

In order to evaluate the potential safety of compounds extracted to mammalians, toxicity assays were conducted. Splenocytes from Golden Syrian hamsters were collected and plated in RPMI-10 (RPMI medium supplemented with 10% heat inactivated fetal calf serum, penicillin/streptomycin and L-glutamine) in a 24-well plate. Cells were then exposed to 50µg/mL of the pure *Dalea parryi* compounds. The positive control used was 5mM menadione, This compound has been shown to be toxic to cells by inducing DNA damage via reactive oxygen species formation.[16] 2% DMSO was used negative control in the toxicity evaluations. Samples and controls were run in triplicate. Each well contained 10^6 splenocytes. The cells were incubated for 24 hours at 37°C, 5% CO_2 and then prepared for flow cytometry. They were stained with annexin V, Alexa Fluor 488 conjugate and propidium iodide (Thermo Fischer Scientific, Waltham, MA). Stained cell parameters were acquired by flow cytometry using the Bio-Rad S3e Cell Sorter (Bio-Rad Laboratories, Hercules, CA). The flow cytometry data were analyzed and analyzed using the FlowJo software program (Treestar, Ashland, OR).

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