ANTHELMINTIC POTENTIAL OF PLANT EXTRACTS ON HELMINTHS IN SMALL RUMINANTS

by

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DEDICATION

I would like to dedicate this thesis work to my mom, Janet Luce. The unwavering support she provided through my education and trialing health was essential for the completion of this thesis and my success through college.

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LIST OF ABBREVIATIONS

Abbreviation	Description
GIN	Gastrointestinal nematode
AR	Anthelmintic resistance
ML	Macrocyclic lactones
GluCIRs	Glutamate-gated chloride ion channel receptors
ACT	Artemisinin Combination Therapy
PSO	Pumpkin seed oil
FEC	Fecal egg count
BZ	Benzimidazole
WEC	Fecal worm egg count

ABSTRACT

Gastrointestinal nematodes (GIN) cause substantial economic loss in the livestock industry. Currently, many anthelmintic drugs being used to treat GIN fall under only three classes of drugs. This leaves relatively few available alternatives to further control and treat GIN in livestock. Livestock producers, through continuous and non-therapeutic use of anthelmintics, have contributed to rapid development of anthelmintic resistant (AR) GIN. Therefore, it is imperative to develop and evaluate alternative anthelmintic treatments in order to effectively treat GIN and AR GIN. Research into numerous plants and plant extracts with anthelmintic potential have demonstrated promise as effective anthelmintic alternatives. Therefore, it was hypothesized that plant botanicals from wormwood (*Artemisia annua)*, garlic (*Allium sativum)*, and pumpkin seed (*Cucurbita maxima)* will prove to be just as effective as fenbendazole when treating GIN in small ruminants. Allicin exhibited a time-to-death similar to fenbendazole at the highest concentration tested (2.15 ± 0.54 h vs. 1.00 ± 0.54 h, p= 0.1527). *Haemonchus contortus* were exposed to botanical compounds *in vitro* where time-to-death was observed. Wormwood, garlic, and pumpkin seed were evaluated independently at varying concentrations. The current study revealed that allicin and pumpkin seed oil demonstrated significant anthelmintic activity against adult *H. contortus* at the concentrations tested.

I. INTRODUCTION

Gastrointestinal nematodes (GIN) are rapidly developing resistance to commercial anthelmintics and posing a significant economic threat to small ruminant producers around the world (Craig, & Miller, 1990; Artho et al., 2007; Qamar, Maqbool, & Ahmad, 2011). Anthelmintics are estimated to consist of 53% of veterinary drug use worldwide (Diaz Lira et al., 2008). There are only a few classes of anthelmintic drugs used to treat or control GIN in livestock, and their non-therapeutic use has contributed to the exponential rise in resistant GIN (Kaplan, 2004; Cwiklinski et al., 2013). This has resulted in substantial economic loss to many livestock producers around the world (Qamar, Maqbool, & Ahmad, 2011). The symptoms of GIN infection include anemia, retarded growth, reduced productivity, tissue damage, and even death (Nieuwhof and Bishop, 2005; Qamar, Maqbool, & Ahmad, 2011) *Haemonchus contortus,* the most economically significant GIN, feeds on the blood of its host causing additional symptoms that include anorexia, diarrhea, and edema due to the loss of blood and plasma proteins (Jex, & Gasser, 2013; Roeber et al., 2013; Fleming, Scharko, 2015).

The traditional approach to treating GIN involves administering an appropriate anthelmintic at the correct interval. This approach does not consider the interaction the parasite has with the anthelmintic and the environment. Interval treatment does not differentiate infected animals from healthy animals, but instead treats the entire livestock population, regardless of symptoms. This herd treatment method does not utilize symptom observation and fecal egg counts (FEC) to determine an accurate infection status of each individual. This results in healthy animals being routinely treated with anthelmintics, exposing sub-clinical GIN to sub-lethal doses of anthelmintics, which

contributes to GIN anthelmintic resistance. A higher AR GIN population increases the potential of introducing resistant GIN to the external environment, further exacerbating the spread of anthelmintic resistance and subsequent herd infection (Artho et al., 2007). As a result, AR GIN populations are appearing all over the world (Craig, & Miller, 1990; Kaplan, 2004; Artho et al., 2007; Qamar, Maqbool, & Ahmad, 2011). Over the last 20 years, the traditional approach to treating GIN has become outdated and ineffective (Fleming, Scharko, 2015).

A promising, alternative approach to combating the growing number of resistant GIN involves using plant extracts with known anthelmintic properties. *Artemisia annua* (Wormwood), *Allium sativum* (garlic), and *Cucurbita maxima* (pumpkin) have all exhibited anthelmintic characteristics independently (Skyles, & Sweet, 2004; Tariq et al., 2009). Using plant extracts has resulted in decreased use of synthetic anthelmintics (Ferreira, 2009). Botanical efficacy also improves when used in combination, indicating an additive effect may exist (Ferreira, 2009; Katiki et al., 2017).

Therefore, it was hypothesized that plant botanicals from wormwood (*A. annua)*, garlic (*A. sativum)*, and pumpkin seed (*C. maxima)* will prove to be just as effective as fenbendazole when treating GIN in small ruminants. The objective of this study was determining the anthelmintic potential of *A. annua* (wormwood), *A. sativum* (garlic), and *C. maxima* (pumpkin seed) independently against *H. contortus.*

II. REVIEW OF LITERATURE

Anthelmintic Resistance

Anthelmintic resistance has become a grave concern for small ruminant producers. Livestock experience depressed growth, mortality, and lack of effective treatment options against resistant GIN (Gasbarre, 2014). Anthelmintic resistance is an unavoidable consequence of anthelmintic use over time. Through the continued, unregulated use of traditional anthelmintics, resistant parasites began to develop. These resistant parasites are protected from anthelmintics by genes passed down from parasites that survived previous anthelmintic exposure (Wade, 2016). Parasites can be resistant to one or more forms of anthelmintics, making them exceedingly difficult to treat (Howell et al., 2008; Wade, 2016).

Although numerous anthelmintics exist, many of them fall under only three classes of drugs: benzimidazoles, imidazothiazoles, and macrocyclic lactones. Recently, three new classes have been approved for use against GIN in small ruminants to help counter the widespread resistance against the three original anthelmintics (Kaplan, 2004; Cwiklinski et al., 2013). However, due to the similarities between drugs, parasites are often resistant to multiple classes of anthelmintics (Wyk, Malan, & Randles, 1997; Cwiklinski et al., 2013; Wade, 2016).

Anthelmintic resistance first appeared in discrete populations of ruminants in the 1950s and by 1970 had spread globally, with most sheep rearing countries documenting some degree of anthelmintic resistance (Craig, & Miller, 1990; Kaplan, 2004; Artho et al., 2007; Qamar, Maqbool, & Ahmad, 2011). Multiple cross-sectional studies around the world have observed an increase in anthelmintic resistant parasites over time and an

increase in the number of parasites that are resistant to multiple drug classes (Falzon et al., 2014).

Eggs recovered from the feces of four different sheep and goat breeds in Pakistan were resistant to albendazole, indicating the prevalence of resistant GIN throughout multiple breeds (Muhammad et al., 2015). In South Africa, a helminth strain was found to be resistant to the compounds from all anthelmintic groups available in 1997. It was concluded that resistance to anthelmintics in South Africa is moving at a faster pace than our ability to create new anthelmintics to treat resistant GIN (Wyk, Malan, & Randles, 1997). This example provides insight into the degree of resistance that can develop through the overuse of anthelmintics (Wade, 2016).

The infectious nature of resistant GIN was further described in 2007 when a White Alpine sheep farmer purchased Dorper sheep, which were harboring anthelmintic resistant (AR) GIN. The White Alpine sheep were initially free from resistant GIN, but soon after introducing the Dorper sheep, the White Alpine sheep were positive for AR GIN. The Dorper sheep were grazed on the same pasture as the Alpine sheep, leading researchers to hypothesize the Dorper sheep were responsible for contaminating the pasture with AR GIN. Researchers speculate the resistant GIN were derived from the Dorper sheep (Artho et al., 2007). This discovery strongly suggests that AR nematodes are capable of infecting pastures that were once free from AR GIN. This threatens geographical regions that are currently free from resistant parasites.

Anthelmintic resistant GIN are also an emerging issue in the cattle industry. In the last 40 years, the genetic makeup of GIN in the United States has been altered through the survival and reproduction of anthelmintic resistant GIN resulting in GIN

resistance in the cattle industry. For example, studies have shown macrocyclic lactones exhibiting efficacy less than expected in over 15 different states in the US (Gasbarre, 2010). Additionally, anthelmintic resistance was observed in vastly separate regions of the US; the Southeast and Northwest, indicating that AR GIN have the ability to develop independent of existing populations of AR GIN.

Economic Impact

Small ruminant production is an important income source for many small-scale farmers, along with providing meat and milk for many countries in the world (Ahmad et al., 2016). Many regions of the world rely on small ruminants for food, milk, and clothing (Ronchi & Nardone, 2003). The rise of anthelmintic resistance increases economic stress on producers and consumers due to the decrease in production and mortality (Gasbarre, 2014). The lack of options to treat AR GIN and their ability to infect other animals has caused livestock producers to suffer substantial economic loss (Waller, 1997).

In the United States, Ivermectin resistance among field populations of *H. contortus* was first reported by Craig and Miller (1990) in a herd of Angora goats in East Texas. The *H. contortus* found in these animals developed AR nematodes after 5 years of routine treatment, indicating that resistant nematodes have been present in Texas for at least 27 years. The longstanding presence of AR nematodes in Texas strongly suggests that substantial economic losses are being incurred. According to USDA (2013), the Texas sheep inventory was 700,000, and the goat inventory was 850,000. The same report listed national sheep inventory of 5.3 million and goat totals of 2.3 million. In 2012 a census done by the USDA, U.S. sheep, goats, and their products totaled $939.7

million annually. Australia loses 8.7% of its annual production value to GIN (Meat and Livestock Australia, 2006). Therefore, if the U.S. encounters similar losses due to GIN, the potential annual economic impact is as much as $81 million. In other countries, where small ruminant production is the primary source of protein and milk, GIN losses are especially detrimental to the economy.

The symptoms caused by *H. contortus* have been estimated to cause a 27% reduction in body weight and a 29% reduction in milk yield (Qamar, Maqbool, & Ahmad, 2011). *Meat & Livestock Australia* estimated gastrointestinal nematodes cost the Australian sheep industry $369 million annually (Abbott et al., 2006). *H. contortus* has been labeled as the most economically important GIN due to the substantial economic loss it causes to livestock producers around the world. *H. contortus* has cost the countries of Kenya, South Africa, and India $175 million in treatment costs alone (Qamar, Maqbool, & Ahmad, 2011).

In Sri Lanka, where goat husbandry is one of the primary sources of income for the livestock farming population, over 14 percent of the total cost of goat husbandry is spent on the control of parasites (Kothalawala, Fernando, & Kothalawala, 2007). Animal losses due to GIN have been estimated at 39,547.4 kids per year and 1,417.94 metric tons of mutton per year in Sri Lanka. The total monetary loss, not including infertility, veterinary cost, and milk loss equaled 229.48 million Sri Lankan rupees equaling $3,582,182.80 USD (Kothalawala, Fernando, & Kothalawala, 2007). Economic losses due to decreased productivity have also been recorded in cattle in the presence of anthelmintic resistant GIN (Fazzio et al., 2014). In summary, GIN infections consistently have a significant negative impact on livestock production due to mortality and decreased

productivity (Mavrot, Hertzberg, & Torgerson 2015). Traditional anthelmintics have provided producers with a temporary means to combat these losses, but a more sustainable approach needs to be implemented to overcome the growing economic threat resistant GIN present. The use of botanicals as anthelmintics has the potential to alleviate the economic pressure of resistant GIN.

Traditional Anthelmintics

Traditional anthelmintics have routinely served as the first line of defense against GIN infections. Although traditional anthelmintics have proven to be effective their effectiveness has taken a decline as AR parasites make a rise. Traditional anthelmintics are limited to three major classes: macrocyclic lactones, benzimidazole, and Imidazothiazoles. Traditional anthelmintics have historically worked by killing the worm or causing its expulsion without harming the host animal. Research has revealed many of the avenues in which traditional anthelmintics work but have yet to fully understand the genes responsible for resistance.

Macrocyclic Lactones

Many of the traditional anthelmintics used today in ruminant production use macrocyclic lactones (ML) as their active anthelmintic ingredient. Macrocyclic lactones are chemical compounds derived from soil microorganisms introduced in the 1980s as a broad-spectrum antiparasitic agent (Abongwa et al., 2017). Macrocyclic lactones target glutamate-gated chloride ion channel receptors (GluCIRs) in the neurons and muscle cells of nematodes (Cully et al., 1994; Adelsberger et al., 2000). Macrocyclic lactones permanently activate these channels within the nematode, which inhibit neuronal activity and muscle contractility. The activation of these channels results in paralysis and

death of the nematode.

As resistance to ML drugs developed, researchers incorrectly speculated that mutations in the drug target receptors of nematodes could be contributing to AR (Kotze et al., 2014). Therefore, the lack of insight into the cause of resistance among field isolates of parasitic nematodes challenges the future efficacy of ML drugs.

Benzimidazoles

Benzimidazole (BZ), another popular anthelmintic, was first introduced in 1961. Benzimidazoles target and binds with β-tubulin within nematodes. This binding inhibits microtubule polymerization, which destroys cell structure and leads to death of the parasite (Kotze et al., 2014; Abongwa et al., 2017).

The major genetic determinant of BZ resistance in most nematodes species is the single nucleotide polymorphism in the nematode's isotype-1 β-tubulin gene (Kotze et al., 2014). Benzimidazoles are the best understood when it comes to parasite resistance at a molecular level. Having this knowledge has expanded research on detecting resistant populations of parasites and better understanding the causes of resistance.

Imidazothiazoles

Imidazothiazoles target the transmembrane ion-channel nicotinic acetylcholine receptors (nAChRs) of nematodes. Ligand opens this channel receptor and allows Na^{+} and Ca^{++} to enter, creating a physiological response (Kotze et al., 2014). Here they bind to nAChRs on body wall muscles, causing depolarization and contractions that cause paralysis of the nematode, resulting in expulsion from the host (Laing et al., 2013; Abongwa et al., 2017). Coupling between contraction and imidazothiazole receptor opening could change and may lead to the development of resistance (Kotze et al., 2014).

Many genes are suspected to be involved in resistance to, levamisole making it a less understood form of resistance.

Traditional anthelmintics, although once highly effective, have experienced a gradual loss of efficacy as parasites have developed resistance. Though research has been focused on understanding the mode of action and causes of resistance, there are still many unknowns and speculations involved. As resistance develops and traditional drugs lose their efficacy, it becomes more critical to stay ahead of the curve and develop alternatives to the drugs currently being used. With many of the drugs working to inhibit motility and allow the parasite to be expelled from the animal, it gives insight into what characteristics to look for in a potential anthelmintic. Through evaluating different combinations of botanicals that exhibit similar characteristics an affordable alternative to traditional anthelmintics can be developed.

Haemonchus Contortus Life Cycle

H. contortus has a direct life cycle without any intermediate host consisting of two phases; the host phase and the free-living phase. *H. contortus* are roundworms that measure only 30 mm, but in large numbers are capable of consuming up to 10% of the host animal's blood a day (Nixon, 2018). *H. contortus* adults mate and reproduce inside the host animal. The fertilized eggs are then passed into the environment through the feces, where they hatch and develop into L3 infective larvae within 7 days under favorable conditions (Yang et al., 2017). The L3 infective larvae then migrate to the top of forage, where they are consumed by grazing animals (Masamha, Gadzirayi, & Mukutirwa, 2010). This cycle takes 3 weeks to complete, with larvae developing from egg to infective larvae in only 4 to 10 days during the summer months. Due to the rapid

development during the summer months, pasture contamination is rapid in the warmer months, while in winter, larval growth slows substantially. During the winter, larval development to the infective stage takes approximately 1 to 2 months. Infective larvae have a sheath that protects them against harsh environmental conditions. Upon ingestion by the ruminant, the larvae lose this sheath and bury themselves in the mucosa of the abomasum, where they develop into the final larval stage (Fleming, Scharko, 2015). Adult *H. contortus* has been reported to consume 30 μL of blood per day and cause catastrophic damage to the tissue of the abomasum (Dineen et al, 1965).

Fecal egg counts of *H. contortus* rise substantially in the periparturient period. This rise in FEC is sustained at parturition and lasts through lactation. Detecting the dam's weakened immune system, *H. contortus* females can increase egg production up to eight times than what is normally produced and passed through the feces and into the environment (Sargison, Jackson, & Gilleard, 2011; Fleming, Scharko, 2015). This results in a drastic increase in the level of infection of a grazing area.

H. contortus fecundity contributes to its ability to develop rapid resistance. *H. contortus* females have the capacity to produce 5,000 to 15,000 eggs per day. Additionally, *H. contortus* have the shortest patent period and lifecycle of any GIN (Anderson et al., 1978; Emery, Hunt, & Jambre, 2016; Yang et al., 2017). The rapid rise in parasite fecundity during parturition of the ewe allows the parasites that have survived anthelmintic exposure to reproduce and pass on their resistant genes to their offspring (Wade, 2016).

Parasites' ability to evolve contributes to their ability to resist chemical anthelmintics. Through surviving anthelmintic exposure, selection for resistant alleles

will increase throughout the population affecting drug efficacy. Rapid rates of nucleotide sequence evolution, when paired with immensely large population sizes, provide GIN with a plethora of genetic diversity, allowing them to successfully respond to a chemical attack and produce a new generation of resistant GIN. (Blouin et al., 1995; Anderson, Blouin, and Bleech, 1998).

H. contortus fecundity, when paired with their rapid development and immense genetic diversity, creates an ever-evolving adversary to small ruminant producers. Through a better understanding of *H. contortus* life cycle, producers are better prepared to manage a GIN infection strategically. This challenge requires a multipronged approach to mitigate the rise of anthelmintic resistance properly.

Anthelmintic Alternatives

Currently, treatment of GIN relies on the routine use of chemical anthelmintic drugs. However, these drugs have demonstrated decreased efficacy as the global emergence of anthelmintic resistant GIN has occurred (Craig, & Miller, 1990; Artho et al., 2007; Qamar, Maqbool, & Ahmad, 2011; Falzon et al., 2014; Gasbarre, 2014). One promising alternative to traditional treatment involves the use of botanicals or plant-based treatments. Botanicals have shown promise in being an effective, sustainable means of treating GIN (Skyles, & Sweet, 2004; Masamha, Gadzirayi, & Mukutirwa, 2010; Feitosa et al., 2012). Plant-based insecticide and larvicidal compounds are becoming more sought after due to their biodegradable nature and non-toxic effects on other organisms (Bagavan et al., 2008). *A. annua, A. sativum,* and *C. maxima* are three botanicals that have demonstrated anthelmintic activity against GIN. All three botanicals also have a long history of medicinal use.

Artemisia species

A. annua, a plant species in the Asteraceae family, has been used as an anthelmintic since ancient times. The Egyptians documented the medicinal and religious importance of *Artemisia* species in their culture (Skyles, & Sweet, 2004). Along with being known historically as a diaphoretic, A. *annua* has been identified as a potential alternative to traditional anthelmintics. Artemisinin, a compound produced by A. *annua,* has demonstrated effectiveness against Plasmodium, the parasite that causes malaria (Tawfiq et al., 1989).

The effect of *Artemisia* species on parasite mortality has been well documented. *A.* a*bsinthium* CEE significantly reduced EPG by 82.85% and 90.46% when nematodes where treated at 1.0 and 2.0 g/kg BW, respectively (Tariq et al., 2009). Additionally, feeding *A*. a*bsinthium* leaves, fed as 20% in alfalfa pellets throughout the infection and treatment period, significantly reduced parasite burden by 49% and EPG by 73% (Valderrábano et al., 2010). Crude ethanolic extracts of *A*. a*bsinthium* have also demonstrated anthelmintic activity, exhibiting a mean mortality index of 0.95 *in vitro* (Tariq et al., 2009).

The compounds produced by *Artemisia* species have demonstrated efficacy in neutralizing free radicals in the blood of animals that are suffering from GIN infection (Ferreira, 2009). These anthelmintic and antioxidant compounds also assist the body in strengthening the immune system, which in turn assists in clearing gastrointestinal nematode infection. *Artemisia* species have also exhibited a high concentration of sesquiterpene lactones and flavonoids, both of which have demonstrated anthelmintic activity (Ferreira, 2009).

Specifically, the lactones absinthin and anabsinthin have been determined to be the active anthelmintic ingredient in *Artemisia absinthium (A. absinthium)*. Thujone, a terpene within *A. absinthium,* has demonstrated the ability to stun roundworms leading to them being expelled through the feces (Skyles, & Sweet, 2004). These characteristics provide compelling evidence that *Artemisia* species are an effective treatment for GIN.

Allium sativum

Allium sativum (A. *sativum*) - colloquially known as garlic - is another plant with demonstrated anthelmintic properties. It has been used for medicinal purposes for thousands of years, including as an antiseptic to prevent gangrene (Kadam et al., 2015). A. *sativum* has been documented to be an effective parasiticide, larvicide, and immune-stimulant. Specifically, A. *sativum* oil was observed to distort the body surface of helminths, indicating a stress response by the parasite (Shalaby & Farag, 2014). The main biological component in A. *sativum, (S*)-Allyl 2- propene-1-sulfinothioate or allicin, has been identified as contributing to the antiparasitic action (Lima et al., 2011).

Schistosoma mansoni, the parasite that causes Schistosomiasis in humans, is adversely affected by allicin at increasing concentrations. Tegument wrinkling, an indicator of stress, occurred at concentrations as low as 5 mg/mL and ulcers developed on the worm at 20 mg/mL, clearly demonstrating the anthelmintic activity of allicin (Lima et al., 2011). Condensed tannins have also been implicated in *A. sativum's* anthelmintic activity. Condensed tannins directly react with proteins on the external layer of the nematode, and disrupt the normal physiological functions (Masamha, Gadzirayi, & Mukutirwa, 2010).

A. *sativum,* in the form of raw garlic juice, was evaluated for its effects on GIN in

sheep on a farm in Zimbabwe. The garlic juice group exhibited a 0% infection rate for strongyles and 33% infection rate for protostrongyloides, while the commercial anthelmintic Valbazen exhibited a 29.5% and a 50% infection rate for the same nematodes, respectively (Masamha, Gadzirayi, & Mukutirwa, 2010). It was concluded that A. *sativum* could be used as a natural anthelmintic to lower the burden of nematode parasites in small ruminants.

Cucurbita maxima

C. maxima, commonly referred to as pumpkin, belongs to the Cucurbitaceous family that contains several medically important genera. Specifically, pumpkin seeds demonstrated anthelmintic properties when administered to livestock and humans (Grzybek et al., 2016). Pumpkin seeds contain amino acids and terpenoid compounds that are thought to provide the seed with anthelmintic effects. Cucurbitacin could potentially demonstrate nematocidal action on GIN due to its similarities in chemical structure to kainik acid, a nematocidal compound (Marie-Magdeleine et al., 2009). The similarities between chemical structures suggest cucurbitacin could also have a neurodegenerative action on nematodes by substituting for glutamate in a comparable fashion to kainik acid. (Marie-Magdeleine et al., 2009).

In vivo experiments performed on mice indicated that *Cucurbita* species seed extract was effective in reducing both the FEC and the number of adult GIN (Grzybek et al., 2016). Additionally, research with ostriches has demonstrated that pumpkin seeds are effective at reducing GIN. 1 g/kg live weight pumpkin seed decreased egg counts per gram of feces in ostriches. It was concluded that pumpkin seed demonstrates promise as an alternative to anthelmintics currently being used in ostrich farming (Feitosa et al.,

2012).

Pumpkin seeds were examined as a natural anthelmintic in 4-month-old Merino ram lambs. Fecal worm egg counts (WEC) of rams fed pumpkin kernel decreased 65.5% from the initial level of WEC (Strickland et al., 2009). These substantial WEC reductions and consistent anthelmintic efficacy exhibited by *C. maxima* offer hope to a novel treatment against anthelmintic resistant GIN.

Combined Effect

While extensive data has been collected on the anthelmintic properties of *A. annua, A. sativum,* and *C. maxima* individually, data is limited on the combined anthelmintic effects of these plants. Combining anthelmintic plant extracts has the potential to have increased efficacy, which may result in improved treatments (Katiki et al., 2017). When antimicrobials are combined with herbal agents, they suppress microbial resistance. The combining of extracts can also improve oral bioavailability and strengthen the effect of the extracts (Yong et al., 2014). For example, flavonoids used in combination with condensed tannins have exhibited synergistic effects in inhibiting the exsheathment of *H. contortus* larvae (Klongsiriwet et al., 2015). Additionally, creosote bush, paddle cactus, and tarbush extracts demonstrated a synergistic effect on growth inhibition of E. *aerogenes* and S. *typhi* (Vásquez Rivera, Escobar-Saucedo, Morales, NoéAguilar, & Rodríguez-Herrera, 2014). Therefore, evidence exists suggesting that combining plants with anthelmintic potential will increase their overall anthelmintic activity.

Summary

Anthelmintic resistant GIN have become an economic challenge to livestock

production, especially small ruminant production. Limited commercial treatments and overuse have contributed to the rise of AR GIN. Therefore, there is ample opportunity to develop and evaluate potential alternative treatments.

Plant extracts with anthelmintic potential are a possible alternative to traditional anthelmintic treatments. *A. annua, A. sativum,* and *C. maxima* have been utilized in both human and animal medicine to eliminate GIN in varying capacities. Therefore, it is hypothesized that plant botanicals from wormwood, garlic, and pumpkin seed *will* prove to be just as effective as fenbendazole when treating GIN in small ruminants. The objective of this study was to determine the anthelmintic potential of *A. annua* (Wormwood), *A. sativum* (garlic), and *C. maxima* (pumpkin seed) independently against *H. contortus.* The results of this study will further advance the foundational knowledge of natural anthelmintics in agriculture and offer insight into the use of plant extracts as potentially effective anthelmintics.

III. EXPERIMENTAL PROCEDURES

GIN Collection

The abomasa, required for research, were collected from sheep and goats slaughtered at the Mercantile Meat Company in Utopia, TX. *H. contortus* were collected from the abomasum compartment of the rumen. The worms found in the abomasum are often full-grown and sexually mature, limiting their lifespan once an animal is processed. Therefore, fresh abomasa were collected from a USDA inspected slaughter facility and dissected within 5 hours of collection. Upon slaughter, abomasa were immediately placed in a plastic bag and into an ice chest. Once the desired number of abomasa were collected, the abomasa were transported form Utopia, Tx to Texas State University, where dissection and collection of the parasites were performed. Worm collection was carried out using procedures modified from Gamble & Zajac (1992) and Eguale et al., (2007). The abomasa were removed from the storage container and laid flat on a dissection tray. Abomasa were then recognized and separated with string from the omasum and duodenum. The separated abomasum was then moved to the total contents tub to be lanced open and washed out. The abomasum was then opened along the curve, and the chyme emptied into the total contents tub. The chyme was gently washed out of the abomasum with purified water at room temperature while paying close attention to the folds. Everything that was washed out of the abomasum was stored in the total contents tub and discarded. The abomasum was washed free from the majority of loose chyme and laid out on a clean tray. The washed-out abomasum was then carefully inspected for adult *H. contortus*. Abomasa that were free from *H. contortus* were placed in a sealed container and froze until discarded. Abomasa that had visible adult *H.*

contortus were separated, and a sterile dissection needle was used to pick the adult nematodes from the abomasa. Once each nematode was collected, it was gently rinsed with purified water. The nematodes were then gently washed off the dissection needle and into the tray for observation. This process was repeated until the desired number of nematodes were collected.

Treatments and Experiments

Abomasa were generally collected between 10:00 am and 12:00 pm. Following the slaughter of the animal, the abomasum and connected gastrointestinal tract were harvested and sealed in an ice chest at room temperature. Once the desired number of abomasa were acquired, they are secured and transferred from the slaughter facility to the lab where the extraction of *H. contortus* occurred. The extraction technique followed the modified method stated in the text. The collection was accomplished within a 5-hour window of the animals' death. Prior to collection, the 6-well plates were prepared with the varying treatments of botanicals and controls.

Once the specific gastrointestinal nematode, *H. contortus* was collected and the predetermined number of specimens reached, distribution into the prepared treatment wells followed. First, the varying treatment concentrations along with the controls were applied to the 6-well culture plate (Costar). Following the application of the treatments to the wells, three adult *H. contortus* nematodes were distributed into each well of a 6-well plate (Costar) with a lid. Following the separation of the 18 worms into the 6 -well plate, the plate was immediately placed in the incubator (HERATHERM IMC 18) at 37 °C. Observation of the worms was taken at hour 0, followed by increments of 1 h for a total of 10 h. The observer monitored the motility of *H. contortus* nematodes and

recorded findings. Motility status was determined over a five-second viewing with three subsequent observations to confirm the complete loss of motility. Complete loss of motility was identified by no head, tail, or pharyngeal movements over the five second observation.

Artemisinin, Allicin, and pumpkin seed oil were utilized as treatments. All botanicals were purchased from Sigma-Aldrich, St. Louis, Missouri. Each of the three botanicals were tested at differing concentrations to assess the therapeutic range of the extract. Treatments were comprised of a total volume of 1.5 mL with 2% DMSO in physiological saline (8.5-g NaCl/L) being used as the solvent. Artemisinin and allicin were used in their pure form, while cucurbitin was evaluated via pumpkin seed oil. The negative control consisted of 2% DMSO and 1.7 mL of fenbendazole at 100 mg/mL was the positive control.

Statistical analysis

The PROC GLM in SAS was used for ANOVA analysis. Significance was defined as $p<0.05$. Least squared means was used to compare means between groups when statistical difference was detected.

IV. RESULTS

Artemisinin

The effect of artemisinin on *H. contortus* is summarized in Figure 1. No significant effect on time-to-death was observed for the evaluated artemisinin treatments when compared to the negative control 2% DMSO (p>0.05).

Table 1. Artemisinin Tested Concentrations Tested concentrations of artemisinin and labels that correspond with Figure 1.

LABEL	CONCENTRATION
A	0.0285
B	0.0114
C	0.00731
D	0.00571
E	0.00365
F	0.00114
G	0.000456
H	0.000281
I	0.000228
J	0.000141
K	0.0000456
L	0.0000182
M	0.0000112
N	0.0000091
O	0.00000562

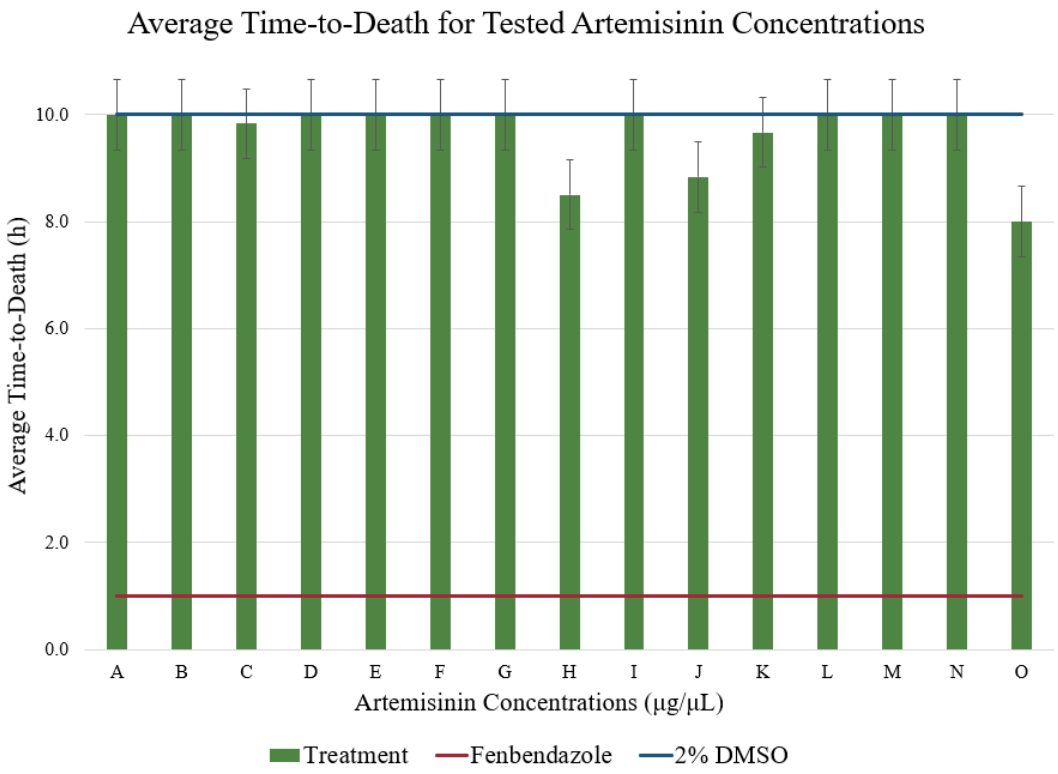


Figure 1. Average Time-to-Death for Tested Artemisinin Concentrations

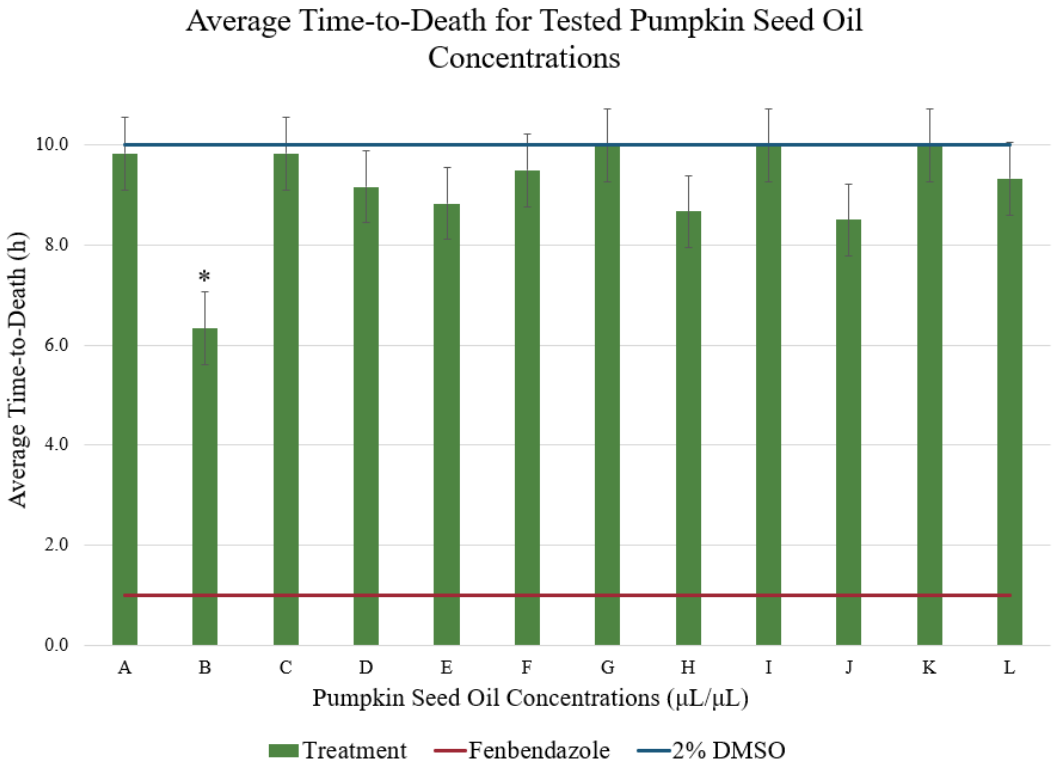
Average time-to-death for *H. contortus* adult nematode exposed to various concentrations of artemisinin.

Pumpkin seed oil

As displayed in Figure 2, concentration B exhibited accelerated time-to-death when compared to the negative control (6.3 ± 0.73 h vs. 10.00 ± 0.73 h, $p < 0.01$). No other evaluated pumpkin seed oil treatment affected time-to-death in the current study (Figure 2).

Table 2. Pumpkin Seed Oil Tested Concentrations Tested concentrations of pumpkin seed oil and labels that correspond with Figure 2.

LABEL	CONCENTRATION
A	0.00459
B	0.00231
C	0.00385
D	0.00192
E	0.000184
F	0.0000922
G	0.000148
H	0.000074
I	0.00000735
J	0.00000369
K	0.00000592
L	0.00000296



* = $p<0.01$

Figure 2. Average Time-to-Death for Tested Pumpkin Seed Oil Concentrations

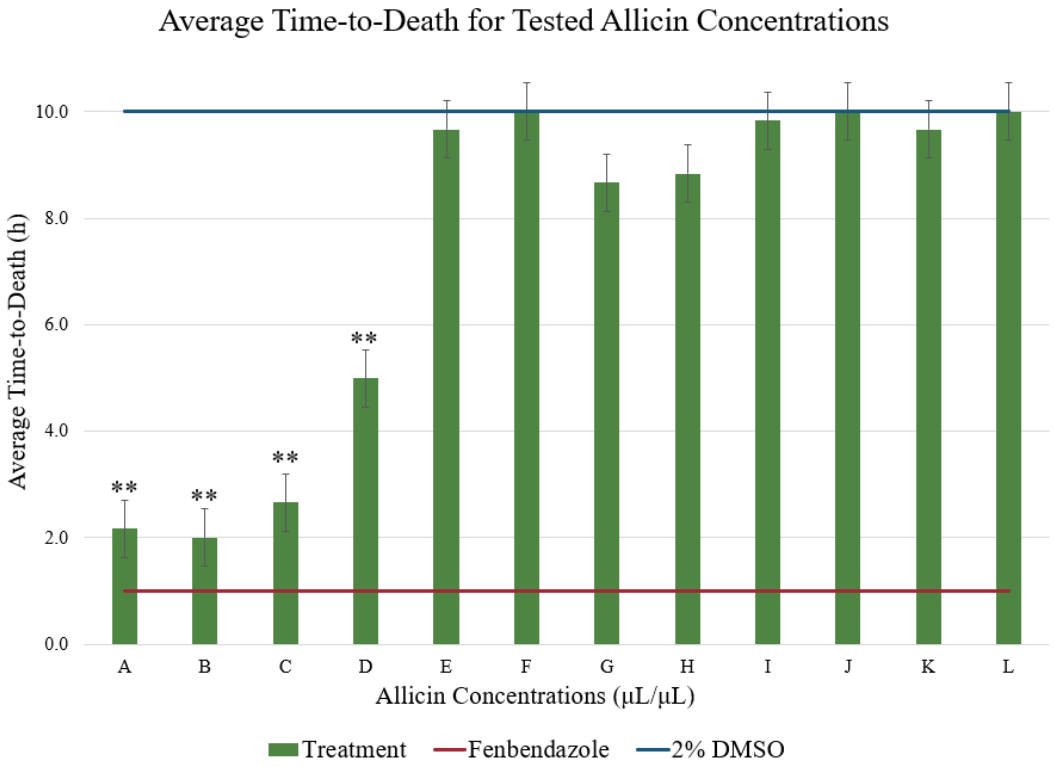
Average time-to-death for *H. contortus* adult nematode exposed to various concentrations of pumpkin seed oil.

Allicin

Allicin significantly reduced the time-to-death at the four highest concentrations tested when compared to the negative control, 2% DMSO (Figure 3, $p<0.0001$). Allicin exhibited a time-to-death similar to fenbendazole at the highest concentration tested (Figure 3, 2.15 ± 0.54 h vs. 1.00 ± 0.54 h, $p= 0.1527$).

Table 3. Allicin Tested Concentrations Tested concentrations of allicin and labels that correspond with Figure 3.

LABEL	CONCENTRATION
A	0.00459
B	0.00231
C	0.00385
D	0.00192
E	0.000184
F	0.0000922
G	0.000148
H	0.000074
I	0.00000735
J	0.00000369
K	0.00000592
L	0.00000296



** = $p<0.0001$

Figure 3. Average Time-to-Death for Tested Allicin Concentrations

Average time-to-death for *H. contortus* adult nematode exposed to varying concentrations of allicin.

V. DISCUSSION

Artemisinin

Published reports of the anthelmintic effects of *Artemisia* species on nematodes guided the current research into experimenting with varying concentrations of artemisinin to establish an *in vitro* concentration that is effective against *H. contortus.* The results of the current study revealed that the concentrations of artemisinin used were not effective in reducing the time-to-death when compared to the negative control, 2% DMSO (Figure 1, $p>0.05$). Previous work suggests that the concentration of artemisinin may not be the exclusive anthelmintic molecule acting within *Artemisia* species (Cala et al., 2014). Artemisinin may prove to have greater anthelmintic activity if used as a whole plant product where all compounds within the plant have an opportunity to work in conjunction against *H. contortus* (Tariq et al., 2009). Natural occurring flavonoids in *Artemisia* species have also demonstrated anthelmintic activity (Kerboeuf et al., 2008).

Additionally, there are other beneficial compounds within *Artemisia* species that can eliminate free radicals and help strengthen the animal's immune system, providing the animal with a higher probability of clearing a parasitic infection (Ferreira, 2009). For example, prior studies on *H. contortus* have confirmed the effectiveness of feeding botanicals and the bioactive compounds that naturally occur within them (Idris, Adam, & Tartour, 1982; Valderrábano et al., 2010). Therefore, incorporating all the compounds present in the plant could be the most effective strategy of utilizing *Artemisia* species as anthelmintics. The flexibility of using and feeding the whole plant product offers producers a simple, affordable, effective treatment that has a minimal risk of mammalian toxicity (Kerboeuf et al., 2008). Thus, using the whole plant as an antiparasitic

supplement in coordination with a traditional deworming schedule could offer a means of combating AR while strengthening the herd's immune health (Ferreira, 2009).

It is plausible that the artemisinin concentrations used and the exposure time in this study were too insignificant to elicit a biological response. In gerbils artificially infected with *H. contortus*, the concentrations of artemisinin evaluated (200 mg/kg BW for 5 days or 400 mg/kg BW single dose) did not produce a significant anthelmintic effect against *H. contortus* nematodes (Cala et al., 2014; Squires et al., 2011). This study reflects the results of our study and highlights the difficulty in standardizing artemisinin's anthelmintic potential. The low solubility of artemisinin in both polar and non-polar solutions is another therapeutic constraint which greatly contributes to the low concentrations of artemisinin used in the current study (Muangphrom et al., 2016). This constraint has led to synthesized artemisinin derivatives being used that have higher solubility (Muangphrom et al., 2016). Recognizing the constraints of plant-based artemisinin provides an opportunity for a combined approach that incorporates existing therapies. In the treatment of malaria, artemisinin has been combined with other conventional antimalarial drugs and proven to have greatly increased parasite cleanse rates in patients (Li et al., 1984). This strategy, known as Artemisinin Combination Therapy (ACT), is the first-line treatment for malaria to prevent relapse and the development of resistance (Muangphrom et al., 2016). Therefore, there is potential to combine artemisinin with current conventional anthelmintics in order to develop a novel approach to combating AR parasites.

Pumpkin seed oil

Prior studies have repeatedly demonstrated the efficacy of *C. maxima* seeds on

reducing the number of GIN and their eggs (Marie-Magdeleine et al., 2009; Grzybek et al., 2016). The anthelmintic potential of *C. maxima* seeds directed the current research into experimenting with varying concentrations of *C. maxima* seed oil to establish an *in vitro* concentration that is effective against *H. contortus.* The results of the current study revealed that pumpkin seed oil significantly reduced the time-to-death at the second-highest concentration tested when compared to the negative control 2% DMSO ($p<0.01$). These results revealed that pumpkin seed oil has the capacity to influence the time-to-death of *H. contortus*. Although statistically significant, the lack of a dose-dependent treatment effect indicates this could be an anomaly. The presence of this potential anomaly urges for a repeated study to evaluate the effective anthelmintic concentration of pumpkin seed oil. Other studies attesting to pumpkin seeds anthelmintic nature used the whole seed (Strickland et al., 2009; Feitosa et al., 2012). Through further evaluation, pumpkin seed oil may not offer the same anthelmintic potential *in vitro* as *in vivo*. A prior study suggested cucurbitine from pumpkin seeds produced aesthesia of the distal proglottids of a tapeworm (TiaoYing et al., 2012). Observations from this study exposed that pumpkin seed might not kill the parasite but help expulsion. Therefore, pumpkin seeds' anthelmintic potential could be evident *in vivo* where expulsion can be considered. Another area that could have affected the current study is the cucurbitacin present in the pumpkin seed oil used. Cucurbitacin, a potential anthelmintic compound, was not measured in our PSO. Future research should investigate the concentration of cucurbitacin present prior to experimentation. Another opportunity to further investigate is the different strains of pumpkin to identify which strains contain the highest concentrations of cucurbitacin.

It is plausible that the concentration of cucurbitacin in the PSO used in the current study was too insignificant to trigger a decrease in time-to-death of *H. contortus* in all but one trial. Although many studies confirmed the effectiveness of *C. maxima,* a study using ground pumpkin seed, pumpkin seed drench, and pumpkin seed oil were ineffective in reducing FEC (Matthews et al., 2016). Therefore, due to the inconsistencies of *C. maxima* when compared to similar studies, the presence of cucurbitacin could be a contributing factor in the effectiveness of *C. maxima.* This knowledge encourages the idea of cucurbitacin being used as an extract and independently tested against *H. contortus.* Cucurbitacin similar structure to a known nematocidal compound, kainic acid could have neurodegenerative action on nematodes (Marie-Magdeleine et al., 2009). Establishing the anthelmintic potential of cucurbitacin could help open the door to further understanding and recognizing structures that have prospective nematocidal action.

Allicin

The traditional use of garlic as an antiseptic and reportedly effective anthelmintic focused this research into establishing an effective concentration of allicin to properly evaluate its potential as an anthelmintic (Masamha, Gadzirayi, & Mukutirwa, 2010; Kadam et al., 2015). The current study revealed allicin demonstrated a dose-dependent treatment effect having a significant effect on time-to-death of *H. contortus* at the four highest concentrations tested when compared to the negative control 2% DMSO ($p<0.01$). These results concur with Kadam (2015), who tested an aqueous extract of *A. sativum* on Indian earthworms. This study concluded that *A. sativum* showed efficacious anthelmintic activity in a dose-dependent manner.

The current results demonstrate allicin's potential as an anthelmintic alternative.

Further research should incorporate feeding trials using allicin to better understand how these dosages respond *in vivo*. Allicin content can range from 0.81 to 3.01% per bulb (Wang et al., 2014). Understanding the low percent of allicin present and the variability per bulb can offer another set of problems in creating a consistent *in vivo* study. Therefore, research into high allicin yielding species of garlic could offer more consistent and effective studies. Prior research has shown allicin to be less effective when used *in vivo* (Velkers et al., 2011). This could have to do with the delivery method of allicin to the abomasum. Research using copper oxide wire particles used gelatin capsules to effectively deliver the treatments to the abomasum (Schweizer et al., 2016). This could be an effective means of delivery for allicin and might impact its effectiveness *in vivo.* Follow up studies should also evaluate the ovicidal and larvicidal effects of allicin when sprayed on forages infected with GIN. Through incorporating an approach that puts the targeted nematode in direct contact with allicin, we can maximize allicin's anthelmintic potential and emulate our *in vitro* results in a practical application.

VI. CONCLUSION

In the current study, allicin and pumpkin seed oil demonstrated anthelmintic activity against adult *H. contortus* at some tested concentrations. Both plant extracts have the potential for working alongside traditional anthelmintics or stand alone to assist in GIN control in small ruminants. Future research will include the evaluation of the synergistic effects of allicin and pumpkin seed oil used in combination as well as the evaluation of *in vivo* delivery strategies. The anthelmintic potential of pumpkin seed oil and allicin demonstrated the potential for the development of an alternative anthelmintic product to offer small ruminant producers struggling with GIN. Continued investigation and refinement of the use of allicin and pumpkin seed oil will yield novel treatment methods for combating GIN and AR GIN in small ruminant populations.

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