DETERMINING THE OVICIDAL CAPABILITIES OF ALLICIN, S-ALLYL

CYSTEINE, AND PUMPKIN SEED OIL FOR MITIGATION OF

HAEMONCHUS CONTORTUS INFECTIONS IN GOATS.

by

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LIST OF ABBREVIATIONS

Abbreviation	Description
BSS	Balanced salt solution
FEC	Fecal egg count
LB	Lysogeny broth
NC	Negative control
PC1	Positive control containing Ivomec
PBS	Phosphate-buffered saline solution
PSO	Pumpkin seed oil
SAC	S-allyl cysteine

ABSTRACT

Anthelmintic drugs are widely used treatment options to control gastrointestinal nematodes found in ruminants worldwide. These gastrointestinal nematodes are becoming increasingly resistant towards these drugs, prior to novel anthelmintic development. One of those parasites, *Haemonchus contortus*, has proven resistant to available anthelmintics, developing new mechanisms of resistance at a rapid pace. This resistance has led to major economic losses worldwide due to haemonchosis. In an attempt to determine athelmintic alternatives and help decrease developing resistance, researchers and producers are turning toward botanical alternatives with documented anthelmintic properties. For this study, it was proposed that plant extracts derived from garlic (allicin, S-allyl cysteine) and pumpkin seeds (PSO) would exhibit ovicidal capabilities *in vitro,* when *H. contortus* eggs were exposed to these extracts. Egg hatch inhibition tests were conducted using these plant extracts. Allicin and S-allyl cysteine exhibited the strongest ovicidal effects at a 4% v/v concentration and 8.7% v/v concentration, respectively. Allicin at the 4% concentration averaged 55% of eggs inhibited from hatching over eight days, while S-allyl cysteine at an 8.7% concentration averaged 43% of eggs inhibited. PSO showed modest inhibition over eight days at the 4% concentration, averaging 24% of eggs inhibited from hatching. These plant extracts could benefit producers needing viable alternatives to synthetic anthelmintics for mitigating anthelmintic-resistant *H. contortus* infections in their goat herds.

Keywords: Anthelmintics, Resistance, Haemonchus, Ovicidal

I. INTRODUCTION

Gastrointestinal nematodes are the main cause of global economic losses in sheep and goat production, and anthelmintics are the most common method chosen for controlling these types of parasites (Prichard 1994; Miller et al. 2012). Currently, the four main classes of anthelmintic treatments include benzimidazoles, imidazothiazoles/tetrahydroxy pyrimidines, macrocyclic lactones, and amino-acetonitrile derivates. Presently, many implemented treatment plans focus on reducing the intensity of infection and transmission of the parasite, rather than completely eliminating the infection. This protects the health of livestock while also minimizing losses in production (Levecke et al. 2012). The introduction of an effective anthelmintic therapy regimen lasts two to ten years before biological mechanisms of resistance assert themselves. This results in a significant portion of the parasite population becoming tolerant to drug doses originally demonstrated to be lethal (Wolstenholme et al. 2004; Scott et al. 2013; French 2018). Despite the increasing resistance to synthetic anthelmintics, repeated dosing of livestock with these medications remains the habitual method used for parasite control, with little development of alternative strategies (Babják 2018). The repeated dosing is the contributory factor to anthelmintic resistance, as the susceptible genomes are removed, leaving only the non-susceptible. Some estimates suggests that costs associated with novel anthelmintic research and development range from $30 to $230 million, with the process easily taking over ten years to complete (McKellar 1994; Witty 1999; Waller 2006).

There are several examples of how the response of an anthelmintic within the host influences the development of resistance in a parasite population. These include

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repeatedly underdosing or improper administration of anthelmintics, as well as enhanced

drug metabolism by some ruminants, like goats (Sangster and Gill 1999). The first cases

of anthelmintic resistance were reported in sheep during the 1950s that were infected

with *Haemonchus contortus*. By the 1990s, *H. contortus* was exhibiting multi drug

resistance (Kaplan 2004). *Haemonchus contortus* infects the gastrointestinal tracts of

small ruminants, and is responsible for outbreaks in disease resulting in sudden death,

costing billions in economic losses. The highest concentrations of *H. contortus*

populations are found in the tropics and subtropics, although the parasite is now

responsible for economic losses in temperate climates (Troell et al. 2006; Besier et al.

2016). *H. contortus* is pervasive, with an establishment rate in single infections in

unsuspecting recipients of approximately 60%, and feeds on the blood of its host (Emery

et al. 2016).

This infection rate results from the female worm's ability to produce between

5,000 and 10,000 eggs daily, resulting in a rapid contamination of pastures (Holmes

1987). Further, *H. contortus* also lives the shortest lifecycle than any other

gastrointestinal nematode. The parasite completes its entire lifecycle in roughly 20 days,

with a prepatent period of only 15 days. Other gastrointestinal parasites found in the

abomasum, like *Teladorsagia circumcincta* (formerly *Ostertagia circumcincta*) have a

comparable life cycle, but female *T.circumcincta* are less prolific with a longer prepatent

period of 21 days, and only produce an average of 100-200 eggs daily. Furthermore,

T.circumcincta does not feed primarily on blood (Zajac 2006; Roeber et al. 2013).

Cooperia curticei, *Nematodirus spathiger*, *N. fillicollis*, *N. abnormalis* and

Oesophagostomum venulosum are common parasites of the small and large intestine, but

have a low pathogenicity compared to *H. contortus* (Zajac 2006). During multiple

attempts to treat infected animals, some *H. contortus* isolates have proven resistant to all

major categories of anthelmintics (Chagas et al. 2013; Irum et al. 2014; Raza et al. 2016;

Dixit et al. 2018; Sangster et al. 2018). With a shorter prepatent period combined with a

high egg production, genetic mutations are occurring at quicker rate than other species,

increasing resistance towards synthetic anthelmintics. Anthelmintics have been reported

to carry prohibitive costs for producers in developing regions. Anthelmintics can also

vary in quality in some countries, posing an environmental hazard and possibly leaving

food residues in meat products (Grade et al. 2008).

Proponents of organic livestock production cite this as a potential problem that

arise from synthetic anthelmintic treatments. Additionally, synthetic treatments may

cause toxicity if improperly administered, and some anthelmintics are known to cause

spontaneous abortions when used to treat infected females that are pregnant (Sackey et al.

1991; Assefa et al. 2018; Wasso et al. 2020). Synthetic anthelmintics often function on a

single mode of action when mitigating *H. contortus* infections, while it is believed that

many plants harbor several complex chemical structures that elicit multiple responses

(McGaw and Eloff 2008; Abongwa et al. 2017). Multiple mechanisms of action

synergistically treating parasite infections likely makes it more difficult for *H. contortus*

to form resistance against these botanical compounds, with several plant extracts already

documented to be effective at reducing *H. contortus* infections in livestock (Váradyová et

al. 2018). Plant based treatments can be marketed as suitable options for treating

helminths in organic livestock systems, and may provide safer alternatives for treating

female goats that are pregnant (Sackey et al. 1991; Cabaret et al. 2002). There are some

plants with proven anthelmintic properties beneficial to the health of livestock, without directly contributing to nutrition (Waller and Thamsborg, 2004). Many plants have been used for centuries to treat and prevent parasitism of gastrointestinal nematodes, with their purported benefits deeply rooted in ethnoveterinary medicine (Athanasiadou et al. 2007; Qadir et al. 2010). Ethnoveterinary medicine embraces a holistic practice that incorporates beliefs and skills relevant to animal husbandry and general care, with recent efforts focusing on researching the biological activity of these medicinal plants. Prior knowledge of these plants largely stems from cultures with extremely fragile oral traditions used within their medicinal systems, lacking any scientific context explaining why these botanicals relieve parasite infections. This leads to a substantial risk of losing vital knowledge that cannot be regained (McGaw et al. 2020). Garlic is one of the most common medicinal plants used to treat a variety of diseases, and seeds from the fruits of the *Cucurbitacae* family have documented medicinal benefits as well (Grzybek et al. 2016; Buono et al. 2019).

II. LITERATURE REVIEW

Garlic

Garlic has been used as a natural anthelmintic, amebicide, larvicide, and an immune stimulant by many cultures for centuries (Perry 1980; Anthony et al. 2005). Current evidence suggests that thiosulfinates are mostly responsible for the anthelmintic properties of garlic (Reuter et al. 1996; Ankri and Mirelman 1999; Salama et al. 2013). Allicin, the most common thiosufinate found in garlic, is produced when garlic is crushed, reacting with alliin, a non-protein amino acid, and the enzyme alliinase (Lawson 1996; Waag et al., 2010). Also classified as a cysteine protease inhibitor, allicin has shown to greatly reduce sporozoite infectivity, *in vivo,* in plasmodium parasites responsible for malaria, by decreasing the parasite burden in infected mice with blood-stage infections (Coppi et al. 2006). Allicin exhibits a high bioactivity, but is extremely sensitive to temperature and pH, resulting in swift decomposition. When exposed to blood, allicin is metabolized in less than one minute (Lawson and Wang 1993; Lawson 1998; Lawson and Hunsaker 2018). Allicin and several of its metabolites were also undetectable in blood or fecal samples collected from volunteers who consumed a large quantity of chopped, raw garlic (Lawson et al. 1992). Even a high potency of allicin is rapidly metabolized to interact with several metabolic pathways, suggesting the metabolites of allicin are mainly responsible for its therapeutic effects, and that allicin ultimately decomposes after encountering stomach acid in humans, releasing allyl sulfides, disulfides and other volatiles that are presumed to be metabolized (Agarwal 1996; Rosen et al. 2001). One of those metabolites, S-allyl cysteine (SAC), is noted for its anti-inflammatory and anti-cancer effects (Kosuge et al. 2002; Shin et al. 2019; Lee et

al. 2020). One study recorded over 98% bioavailability for SAC in rats, and a 100% bioavailability in mice (Nagae et al. 1994).

Kodera et al. (2002) orally administrated SAC to rats and mice and reported that SAC could be extracted from plasma and blood three hours after administering the dose. Kodera et al. (2002) also discovered SAC had a better stability than allicin after absorption; a half-life of 10 hours was determined, with a clearance time exceeding 24 hours. Given that *H. contortus* feeds on the blood of its host, it might be possible that the parasite actively consumes SAC when their hosts are fed garlic or a related extract. Worku et al. (2009) specifically tested the effects of garlic, *Allium sativum*, on both *Coccidia* organisms and *Haemonchus contortus* to analyze potential anthelmintic capabilities. Goats used in this study receiving varying doses of garlic juice showed a substantial reduction in the fecal egg counts of *Coccidia spp*. However, *H. contortus* did not respond to the garlic juice and only experienced a minimal decline in fecal egg counts. Burke et al. (2009), also found that when goats infected with *H. contortus* and fed whole garlic bulbs or treated with garlic juice failed to exhibit a reduction in fecal egg counts. Several of the goats used in this study still needed an anthelmintic treatment within 14 days after receiving the experimental treatments. Interestingly, Strickland et al. (2009), reported that when feeding sheep a partial diet of garlic cloves, a 64.4% reduction in fecal egg counts occurred, which increased to a 74.5% FEC reduction after receiving treatment, suggesting that garlic possibly imposes residual effects related to reducing fecundity after ingestion. Masamha et al. (2010) also reported comparable fecal egg reductions in sheep drenched with raw garlic juice when compared to a synthetic anthelmintic. Roughly 97% of *H. contortus* and other parasite eggs belonging to the

superfamily *Trichostrongyloidea* were reduced per gram of feces in sheep drenched with garlic juice, compared to a 100% reduction in sheep drenched with the anthelmintic Valbazen. Ahmed and Al-jubori (2020) collected live *H. contortus* worms from the abomasa of recently slaughtered sheep and immediately exposed them to alcoholic and aqueous extracts of garlic. They achieved a 100% killing effect on larva at 50 mg/ml concentrations diluted with phosphate-buffered saline solution (PBS), with worms showing complete immobility at days four and five after exposure, respectively. However, the worms were not rinsed after exposure to treatment to determine if motility would resume. Palacios-Landín et al. (2015), managed to achieve a 68.7% reduction in adult *H. contortus* worms in experimentally infected gerbils that were given an *A. sativum* n-hexane extract. Azra et al. (2019) reported that a methanolic extract of whole garlic bulb achieved a 67% mortality rate of adult *H. contortus* worms collected from the abomasum of infected goats within six hours after exposing the worms. However, this reduction only occurred when the extract was left undiluted. The methanolic extract also inhibited 80% of *H. contortus* eggs collected from hatching when left undiluted. Given these studies, garlic has proven potential to be an anthelmintic alternative offering partial relief from *H. contortus* infections.

Pumpkin Seed Oil

Plants within the family *Cucurbitaceae* have remained a staple in several cultures, touted for their wide range of health benefits. Several pumpkin varieties demonstrate anthelmintic properties and have been historically exploited in both humans and swine (de Queiroz-Neto et al. 1994; Guarrera 1999; Beloin et al. 2005; Mägi et al. 2005; Lans et al. 2007). In ostriches, pumpkin seed oil (PSO) proved as an effective anthelmintic when

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decreasing the worm burden of *Libyostrongylus douglassii, C.struthionis,* and *Libyostrongylus dentatus*, reducing fecal egg counts by 90% (Feitosa et al. 2013). Pumpkin seeds were also beneficial at reducing *Aspiculuris tetraptera* in mice (Ayaz et al. 2015), and when tested *in vitro* against the parasite *Ascaridia galli* from chickens, pumpkin seed oil peaked at 85% mortality in the worms when administered at 75 mg/ml (Abdel Aziz et al. 2018). Strickland et al. (2009), reported a 65.5% decrease in fecal *H. contortus* egg counts during treatment with pumpkin seeds, but also reported fecal egg counts immediately increased after the sheep were weaned from treatment. Contradicting evidence from Matthews et al. (2016) shows that pumpkin seed oil produced no noticeable reductions in fecal egg counts when treated with pumpkin seeds, either fed whole seeds or given PSO. Marie-Magdeleine et al. (2008) reported that a methanolic extract of *Cucurbita moschata* seeds at 1.2 mg/ml achieved a modest 23.3% egg hatch inhibitory effect when tested against *H. contortus* eggs. According to Meenakshisundaram et al. (2017), aqueous extracts of *Cucurbita pepo* accomplished significant egg hatch inhibition when tested on *H. contortus* eggs. At a concentration of 80 mg/ml, *C. pepo* managed to inhibit approximately 90% of *H. contortus* eggs from hatching. Also, when tested in vivo, aqueous extract of *C. pepo* achieved 96% reduction in fecal egg counts in sheep naturally infected, comparable to albendazole tested at seven mg/kg bodyweight which resulted 93% reduction in fecal egg counts. Plants in the family *Cucurbitaceae* have historical evidence as an anthelmintic treatment that can provide relief from infection. Pumpkin seeds can also be utilized as a palatable feed source that can replace other grains used in goat production (Klir et al. 2017).

Ovicidal Activity of Plant Extracts

With limited studies on the ovicidal properties of plant extracts derived from garlic and pumpkin seed oil, there are multiple *in vitro* studies assessing the ovicidal capabilities of other plant extracts. There have been over 10,000 studies to date that assess the ovicidal capabilities of plant extracts. Rojo-Rubio et al. (2019) assessed the ovicidal effects of a hydro-alcoholic extract derived from the fruit and leaves of the *Caesalpinia coriaria*, and achieved 100% inhibition of egg hatching when treating *H. contortus* eggs with this extract at a concentration of 25mg/ml. In a similar study, Castillo-Mitre et al. (2017) achieved 100% inhibition of hatching when a hydro-alcoholic extract made from the leaves of the plant *Acacia cochliacantha* at a concentration of 100 mg/ml. Váradyová et al. (2018) achieved 100% inhibition of egg hatching when *H. contortus* were exposed to a methanolic extract of *Artemisia absinthium* at a concentration of 1024 µg/ml. At the same concentration, a 37.5% reduction in egg hatching also occurred using a methanolic extract of *Chamomilla recutita*. Váradyová et al. (2018) also tested various aqueous plant extracts at a concentration of 1563 µg/ml, and achieved a 88.3% egg hatch inhibition using *Althaea officinalis,* 36.6% egg hatch inhibition with *Fumaria officinalis*, and a 40.4% egg hatch inhibition with *Malva sylvestris*. Cortes-Morales et al. (2019) interrupted the egg hatching process of *H. contortus* by 34.3% using a methanolic extract of the plant *Baccharis conferta Kunth* at a concentration of 50 mg/ml, and the inhibition of egg hatching increased to 50% when the methanolic extract of *B. conferta K.* was increased to a concentration of 62.8 mg/ml. A 90% inhibition also occurred after *H. contortus* eggs were treated with the *B. conferta K.* methanolic extract at a concentration of 124.1 mg/ml. Although it is considered toxic to

livestock, Mendonça Soares et al. (2019) created an aqueous extract derived from the dried leaves of *Senecio brasiliensis*, and achieved a 94% egg hatch inhibition when treating *H. contortus* eggs with this extract at a concentration of 10 mg/ml. Utilizing an aqueous extract of fresh *S. brasiliensis* that was not dried achieved a moderate 73% egg hatch inhibition at a concentration of 5 mg/ml.

Exploiting ovicidal properties of plants and their respective extracts can increase mortality by preventing larval development of parasites after oviposition has occurred. Diminishing egg hatchability can decrease pasture larval counts and reduce transmission of infective larvae (Nathan et al. 2005; Molento et al. 2016; Pineda-Cortel et al. 2019). Reducing pathogenicity can significantly reduce worm burdens and future infections (Abriola et al. 2019). For this study, it was proposed that plant extracts derived from garlic (allicin, S-allyl cysteine) and pumpkin seeds (PSO) will exhibit ovicidal capabilities *in vitro,* when *H. contortus* eggs are exposed to these extracts.

Other Proposed Treatment Methods

The utilization of bioactive forages, particularly forage legumes rich in condensed tannins have been proven to reduce fecal egg counts in small ruminants. Incorporating tannin-rich forage legumes can reduce pasture contamination (Valderrábano et al. 2010). Condensed tannins are known to pass through the animal undigested and to be present in the subsequent fecal samples, which may affect *Haemonchus contortus* egg development. (Terrill et al. 1994). There are a variety of plants with other secondary metabolites that have proven beneficial in treating internal parasitism in goats, sheep, and deer (Min and Hart 1980; Hoskin et al. 1999; Athanasiadou et al. 2005; Gaudin et al. 2019). Researchers suggest that these secondary plant metabolites exert a direct ovicidal and larvicidal effect

on *Haemonchus contortus,* and improve protein availability in the host to maintain an effective immune response towards this parasite (Barry and McNabb 1995). Rotational grazing has been another practice that producers have used in the United States, Australia, and New Zealand (Stromberg and Averbeck 1999). First calves are provided an anthelmintic to decrease the amount of egg shedding that occurs, before moving livestock to a different area of pasture considered safe. Ideally the anthelmintic is provided during the summer months, when egg shedding is at its peak. The previously grazed pasture is allowed to rest to give time for any parasite eggs deposited on the soil to hatch. Larvae that are not consumed by grazing livestock will die off, and after the rest period for a specific period of time, livestock can resume grazing on that area of pasture. However, it is unlikely that pasture larval counts will reach zero, since larvae can persist for 6-12 months, and even survive the winter months (Gibbs 1980; Stromberg et al. 1991; Stromberg and Averbeck 1999). Alternate grazing has also been utilized by producers. To reduce parasitism in small ruminants, larger livestock species that are resistant to *Haemonchus contortus,* like cattle and horses, are allowed to graze a pasture, followed by goats. This method increases acreage production by reducing goat parasitism and eliminates intra-specific competition for forage (Mahieu et al. 2007; Mahieu and Aumont 2008). Utilizing different grazing techniques and bioactive forages provide immediate economical solutions for producers that are experiencing widespread resistance within their herd.

Egg Flotation Techniques

The simplest method to determine if a goat is infected with *Haemonchus contortus* is through the use of fecal egg counts. This method of parasite detection is

recommended to determine both the necessity of a goat requiring treatment, and ensures the appropriate choice of anthelmintic used to dose the animal (Norris et al. 2019). *H. contortus* eggs float best in a fecal float with a specific gravity between 1.22-1.32 (O'Grady and Slocombe 1980). Magnesium sulfate is proven to be a suitable option for fecal float solution, and comparable to the efficacy of common sucrose-based solutions like Sheather's solution (Faust et al. 1938; Hinaidy et al. 1988; Bharkad et al. 1999; Silva et al. 2009). A magnesium sulfate fecal float can be created by heating distilled water, and combining magnesium sulfate with the distilled water until a specific gravity of 1.27 g/ml is reached after the fecal float has been allowed to return to room temperature. In order to ensure a specific gravity of 1.27 g/ml is achieved, a 1 ml sample can be weighed on an analytical balance. Fecal egg counts are a simple yet accurate method of assessing the effectiveness of synthetic anthelmintics as well as plant-based alternatives incorporated in a producer's treatment regimen.

III. OBJECTIVES

For this study, it was proposed that plant extracts derived from garlic (allicin, S-allyl cysteine) and pumpkin seeds (PSO) would exhibit ovicidal capabilities *in vitro,* when *H. contortus* eggs were exposed to these extracts. The objectives of this study is to test the ovicidal effects, *in vitro*, of the following plant extracts: allicin, S-allyl-L-cysteine, and pumpkin seed oil (Sigma-Aldrich, St. Louis, MO).

IV. JUSTIFICATION

Little research has been completed regarding the effectiveness of *A. sativum* and pumpkin seed oil pertaining to *H. contortus*. The compound allicin was chosen for egg hatch inhibition assays since it is the most abundant bioactive organosulfur compound synthesized in *Allium* species, typically accounting for 70% of the total thiosulfinates found in garlic (Rybak et al. 2004; Salehi et al. 2019; Jiang et al. 2020). SAC was chosen for this study since it is a water-soluble organosulfur compound that has nearly 100% bioavailability when absorbed by the gastrointestinal tract (Colín-González and Santamaría 2017). Pumpkin seed oil was chosen for this study since seeds of the *Cucurbita* species are known to contain various secondary metabolites, such as nitrogen-containing compounds (cucurbitacin B, cucurbitin), saponins, amino acids, and sterols which were reported to have ovicidal and larvicidal effects (Gonzales et al. 1974; Maciel et al. 2006; Grzybek et al. 2016).

<center>**V. METHODS**</center>

Animals

Spanish, Boer, and Spanish x Boer goats, raised on pasture and housed at the Freeman Center (San Marcos, TX) were utilized for this study. All animal work was approved under the Texas State IACUC protocol #7228. Fecal samples were collected in a similar manner as the methods described by von Samson-Himmelsterjna et al. (2009) and Mancilla-Montelongo et al. (2019); fecal samples were collected directly from the rectum of donors into a lubricated nylon glove, turned inside out and tied, and labeled with the corresponding animal number. Fresh samples were processed within 24 hours after collection, with fecal samples cooled and stored in a refrigerator after collection until use.

Fecal Egg Counting Procedure

After fecal samples were pooled together, the pellets were mashed in a mortar and pestle, then transferred to a large beaker to be combined with fecal float. Fecal float was prepared by heating distilled water and combining magnesium sulfate until a specific gravity of 1.27 g/mL is recorded, since *H. contortus* eggs float best at a specific gravity between 1.22-1.32 (O'Grady and Slocombe 1980). Specific gravity was measured by weighing 1ml of fecal float solution to ensure a specific gravity of 1.27 g/mL. Magnesium sulfate has been proven to be a suitable option for fecal float solution, comparable to the efficacy of common sucrose-based solutions like Sheather's solution (Faust et al. 1938; Hinaidy et al. 1988; Bharkad et al. 1999; Silva et al. 2009). The mashed fecal sample were combined with magnesium sulfate fecal float solution, then stirred until the fecal sample and fecal float were thoroughly mixed. The fecal mixture

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was allowed to for ten minutes before being filtered through a sifter or strainer to separate

solid debris from the fecal suspension. After the solid debris was removed, the fecal

suspension was filtered through Whattman Filter Paper No. 1 three separate times. The

fecal suspension was centrifuged in a similar manner as the methodologies described by

Egwang and Slocombe (1982), and Dryden et al. (2005), with a few modifications. The

fecal float suspension was placed in 15ml centrifuge tubes until a meniscus formed at the

top of the tube, then was covered using a coverslip. The fecal suspension was centrifuged

at 1100 rpm for four minutes in a swing bucket centrifuge. The tubes were emptied into a

clean beaker, and the suspension was allowed to rest for ten minutes. An initial egg

concentration was calculated by pipetting 500 µl of the fecal suspension from the surface,

and placed on a slide to view the number of *H. contortus* eggs using an EVOS XL Core

Imaging System (Thermo Fischer Scientific, Waltham, MA). The eggs counted in the 500

µl sample were verified to be *H. contortus,* and the volume of fecal float needed to

pipette 100 eggs into each was calculated using cross products to provide a proportionate

ratio.

Treatment Preparations

To ensure at least 100 eggs were plated into each well of a six-well culture plate,

the wells were counted before exposed to treatments. The amount of lysogeny broth (LB)

added to each well (in µl) was the same amount of fecal suspension required to plate 100

eggs into each well. Allicin treatments were diluted using a balanced salt solution (BSS)

to produce three different concentrations: 3%, 6%, and 12% of plant extract to BSS,

similar to the methods described by Alvarez et al. (2009) and Katiki et al. (2011). This

brought the total volume of the allicin/BSS treatment to equal the total volume of fecal

suspension required to pipette 100 eggs into each well. This creates a 1%, 2%, and 4% v/v ratio of allicin and BSS, respectively, after it was mixed in with the fecal suspension and LB. A pumpkin seed oil (PSO) mixture was created in the same manner using BSS as a diluent, and PSO was also tested at a 1%, 2%, and 4% v/v concentration. For S-allyl cysteine, an aqueous mixture was first created by combining 10 mg of SAC powder with one ml of water. Next, the SAC aqueous mixture was diluted in the same manner as the other two compounds using BSS to produce 12%, 18%, 24% plant extract to BSS concentrations. SAC was tested at 4.2%, 6.4%, and 8.7% v/v concentrations when the plant extract and BSS solution was mixed added to the final well volume. A negative control (NC) was created, which was a combination of equal volumes of fecal suspension, LB, and BSS without any plant extracts A positive control (PC1) was created testing Ivomec, a cattle pour-on containing ivermectin. This control was created by combining the same volumes fecal suspension, LB, and Ivomec. A second positive control was tested using equal volumes of fecal suspension, LB, and methanol. Methanol was chosen as a second positive control since Ivomec is an alcohol-based pour on containing ivermectin. Each treatment was replicated twice each trial. Three trials testing allicin and PSO at their three different concentrations were completed. Four trials of SAC were completed.

Egg Hatch Inhibition Tests

After all respective treatments were mixed, the mixtures were allowed to rest for 24 hours. Each well was counted for eight consecutive days, with the number of eggs in the treatment still unhatched recorded.

The formula used to determine the ovicidal activity was one reported by Ceballos et al. (2019), with some modifications:

$$\% \ Ovicidal \ Activity = \frac{100 \ eggs - \# \ eggs \ hatched \ in \ treatment}{100} \ X \ 100$$

Table 1. Allicin/PSO Treatment Formulation

Concentrations	Fecal suspension mixture[1]	Plant extract v/v ratio
1%	$(X_1 + X_2 + X_3) = 3X$	0.01(3X)
2%	$(X_1 + X_2 + X_3) = 3X$	0.02(3X)
4%	$(X_1 + X_2 + X_3) = 3X$	0.04(3X)

Table 2. SAC Treatment Formulation

Concentrations	Fecal suspension mixture[1]	Plant extract v/v ratio
4.2%	$(X1 + X2 + X3) = 3X$	0.042(3X)
6.4%	$(X1 + X2 + X3) = 3X$	0.064(3X)
8.7%	$(X1 + X2 + X3) = 3X$	0.087(3X)

[1] X_1(Fecal Suspension), X_2 (LB), X_3 (Plant extract/BSS) were added into each well at the same volume; when added together they equal the total volume of the well (3X).

Statistical Analysis

Effects of treatment and observation day with their interactions on egg hatch inhibition were determined using the JMP Analysis software (Cary, NC). Treatment means were compared when a significant difference was detected. Significance was defined as $p < 0.05$.

<div align="center">

VI. RESULTS

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Allicin

      The average number of eggs inhibited over the eight-day experimental window for the 1% allicin treatment equaled 41%. The 2% allicin treatment averaged 46% of eggs inhibited from hatching, while the 4% treatment averaged 55% of eggs inhibited from hatching (Figure 1). There was a significant effect of the 1% allicin treatment compared to the NC. This effect first began on day four and continued through until the end of the experiment (Figure 2). There was a significant effect of the 1% allicin treatment compared to the positive control that began on day seven, continuing until the end of the experiment. A significant effect of the 2% allicin treatment compared to the NC that began on day four and continued until the end of the experiment (Figure 3). There was also a significant effect of 2% allicin compared to the PC1 on day eight. There was a significant effect of the 4% allicin treatment compared to the NC that began on day four, and continued until the end of the experiment (Figure 4).
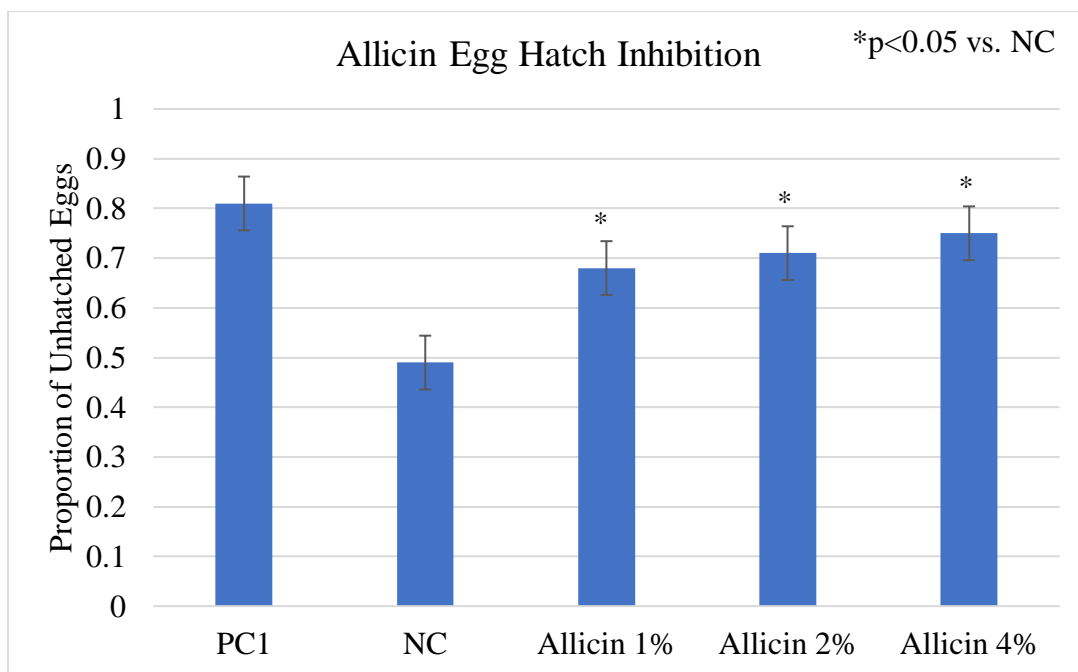
Figure 1. Average effect of allicin concentrations on *H. contortus* egg hatch inhibition over 8-day experimental window. *p<0.05 vs. NC.

Figure 2. Effect of allicin 1% concentration on *H. contortus* egg hatch inhibition over 8-day experimental window. *p<0.05 vs. NC; †p<0.05 vs. PC1.

Figure 3. Effect of allicin 2% concentration on *H. contortus* egg hatch inhibition over 8-day experimental window. *p<0.05 vs. NC; †p<0.05 vs. PC1.

Figure 4. Effect of allicin 4% concentration on *H. contortus* egg hatch inhibition over 8-day experimental window. *p<0.05 vs. NC.

Pumpkin Seed Oil

The 1% PSO treatment averaged 19% of eggs inhibited from hatching over eight-day experimental window. The 2% PSO treatment averaged 23% of eggs inhibited from hatching, while the 4% treatment averaged 24% of eggs inhibited from hatching. There was no significant effect noted for the three concentrations compared to the NC (Figure 5). There was a significant effect of the 1% PSO treatment compared to the PC1 that began on day four and continued until the end of the experiment (Figure 6). The same significant effect was noted for the 2% PSO treatment compared to the PC1 (Figure 7). A significant effect of the 4% PSO treatment compared to the PC1 first began on day five, and continued until the end of the experiment (Figure 8).

Figure 5. Average effect of PSO concentrations on *H. contortus* egg hatch inhibition over 8-day experimental window.

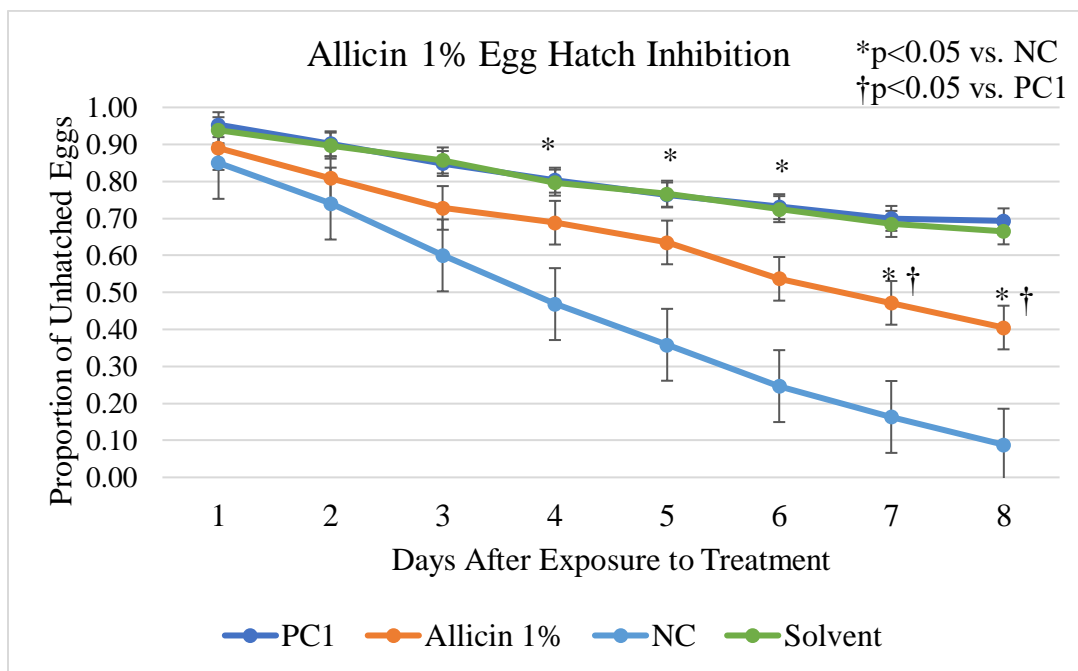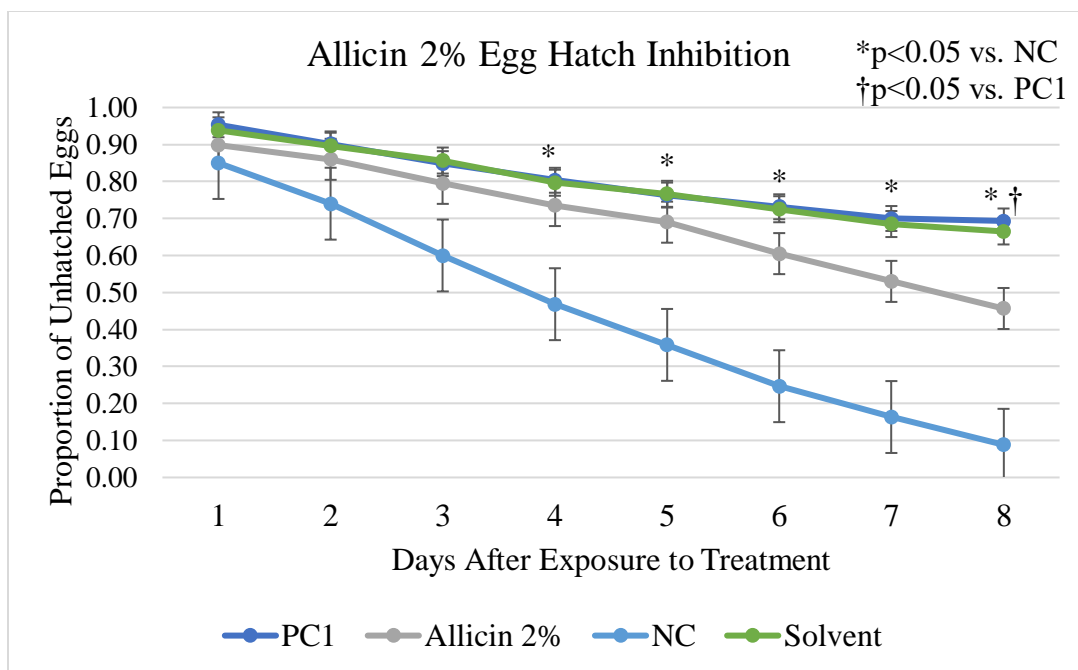**Figure 6.** Effect of PSO 1% concentration on *H. contortus* egg hatch inhibition over 8-day experimental window. †p<0.05 vs. PC1.

Figure 7. Effect of PSO 2% concentration on *H. contortus* egg hatch inhibition over 8-day experimental window. †p<0.05 vs. PC1.

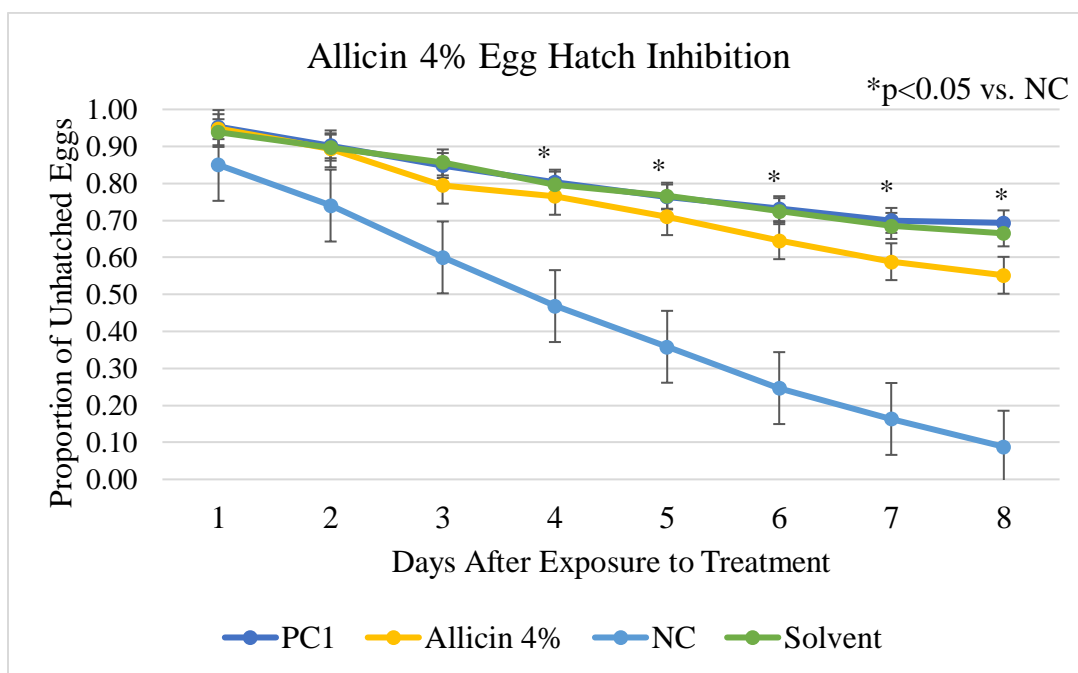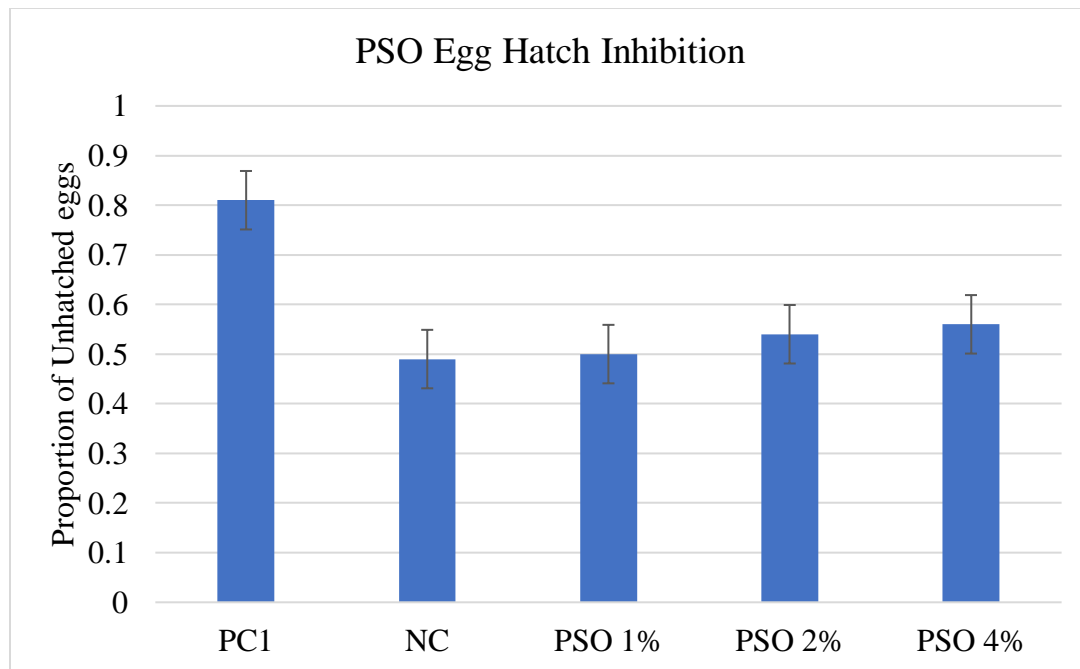**Figure 8.** Effect of PSO 4% concentration on *H. contortus* egg hatch inhibition over 8-day experimental window. †p<0.05 vs. PC1.

S-allyl Cysteine

The 4.2% SAC treatment averaged 36% of eggs inhibited from hatching over the eight-day experimental window. The 6.4% SAC treatment averaged 40% of eggs inhibited from hatching, and the 8.7% treatment averaged 43% of eggs inhibited from hatching. There was a significant effect noted for all three concentrations when compared to the negative control (Figure 9). When assessing that significance by each day, a significant effect of the 4.2% SAC treatment compared to the NC began on day six, and continued until the end of the experiment. A significant effect of the 4.2% SAC treatment compared to the PC1 also began day six, continuing until the end of the experiment (Figure 10). A significant effect of the 6.4% SAC treatment compared to the NC was noted on day three, and continued until the end of the experiment A significant effect of the 6.4% SAC treatment compared to the PC1 began on day six, and continued until the end of the experiment (Figure 11). A significant effect of the 8.7% SAC compared to the

NC began on day three, and continued until the end of the experiment. A significant

effect of the 8.7% SAC treatment compared to the PC1 also began on day six, continuing

until the end of the experiment (Figure 12).
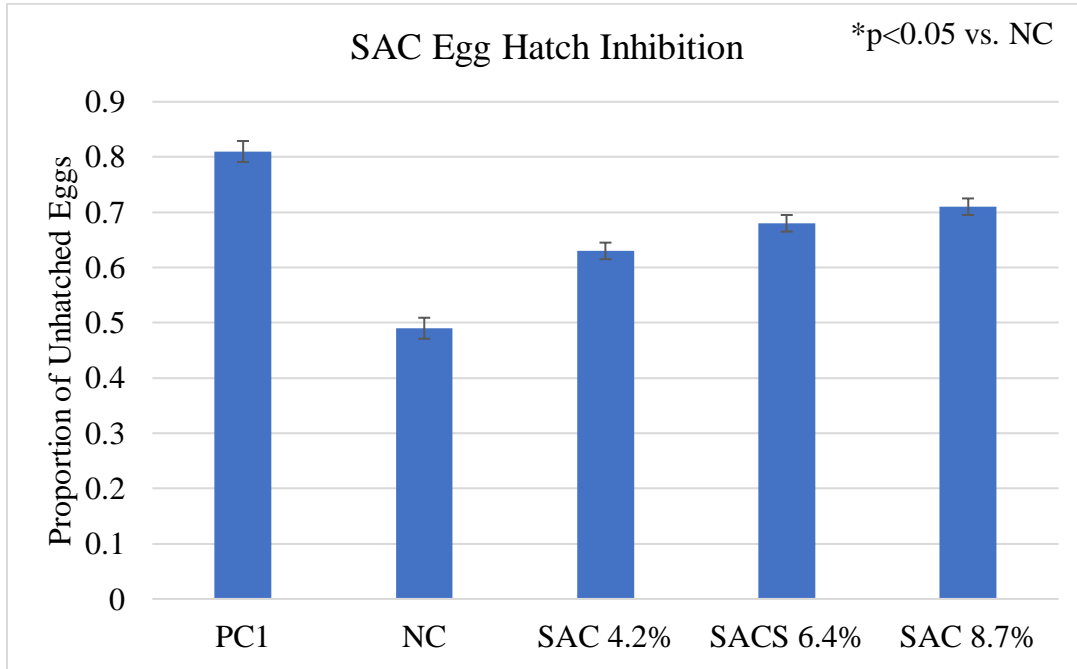
Figure 9. Average effect of SAC concentrations on *H. contortus* egg hatch inhibition over 8-day experimental window. *p<0.05 vs NC.
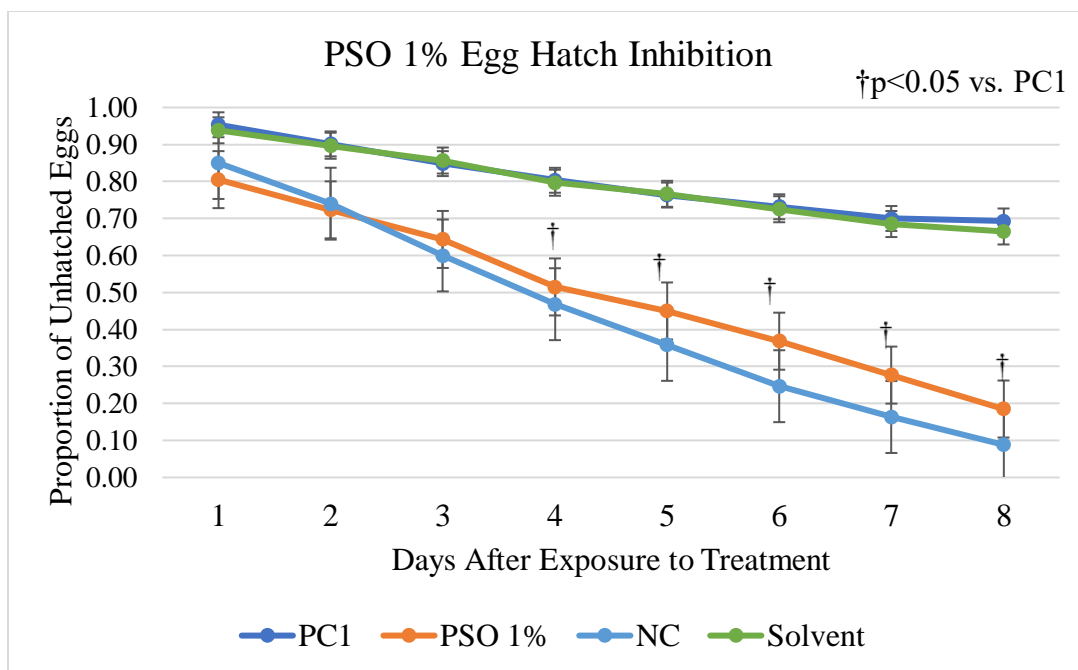
Figure 10. Effect of SAC 4.2% concentration on *H. contortus* egg hatch inhibition over 8-day experimental window. *p<0.05 vs. NC; †p<0.05 vs. PC.
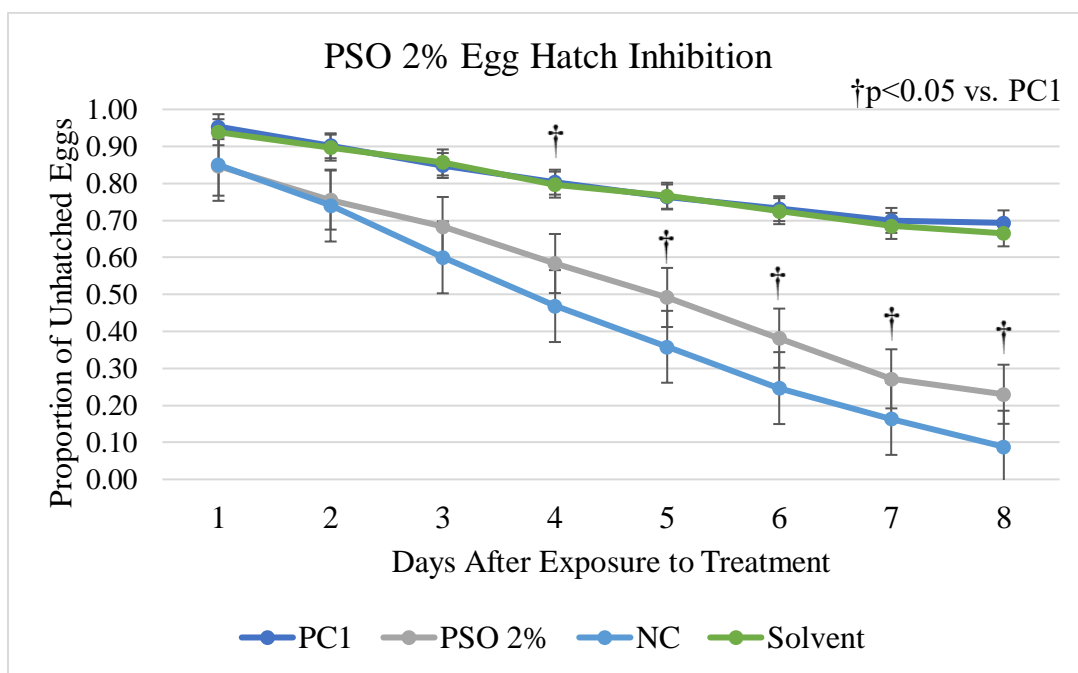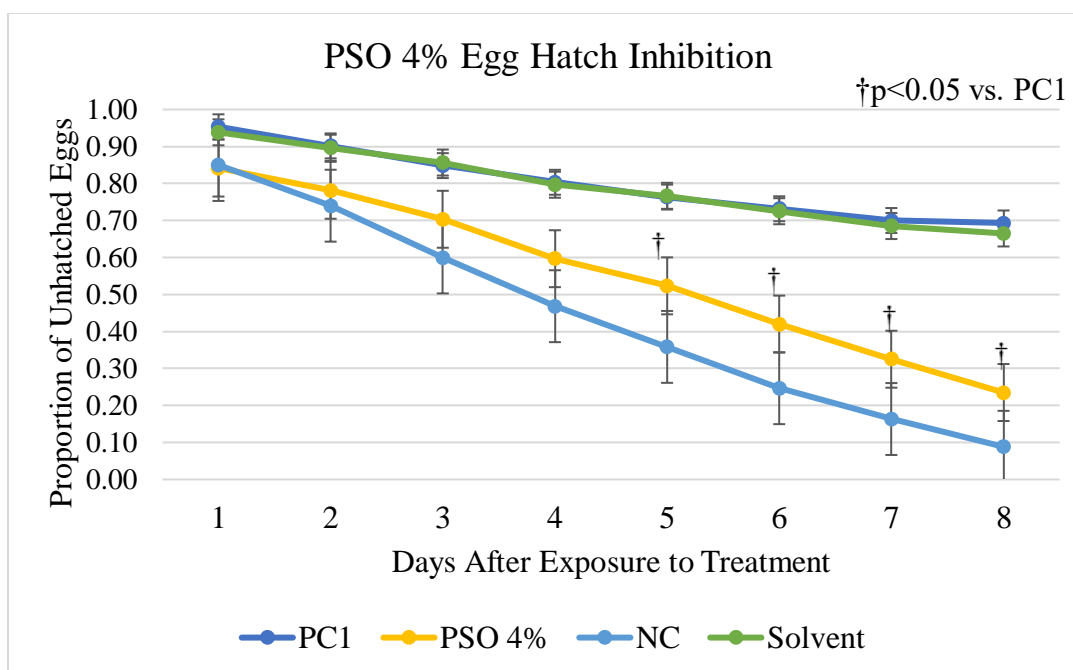

Figure 11. Effect of SAC 6.4% concentration on *H. contortus* egg hatch inhibition over 8-day experimental window. *p<0.05 vs. NC; †p<0.05 vs. PC1.

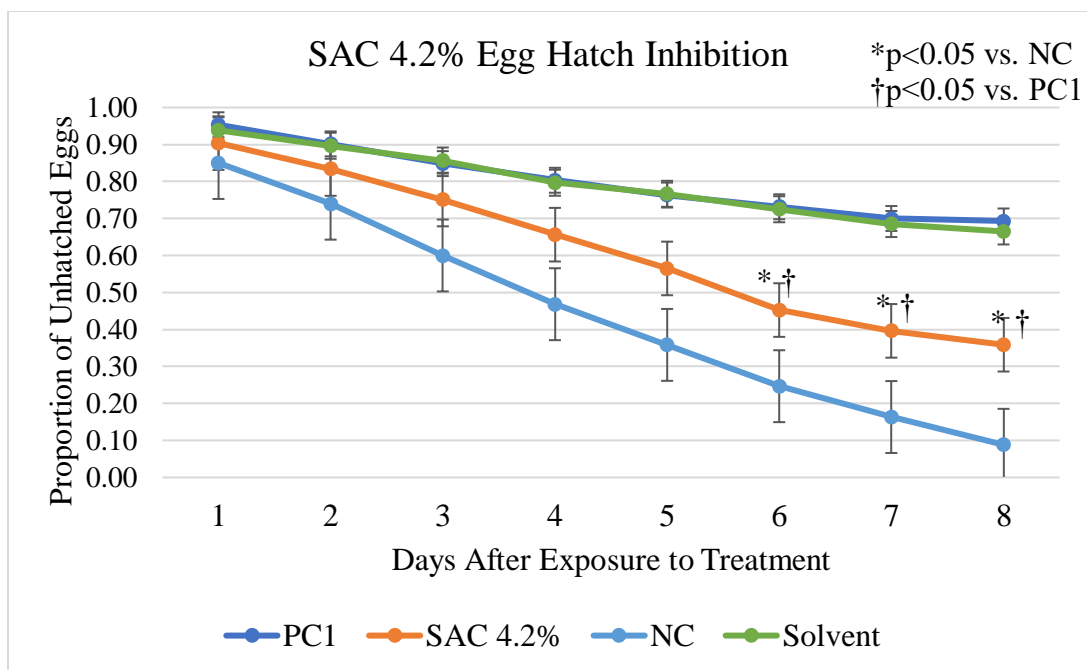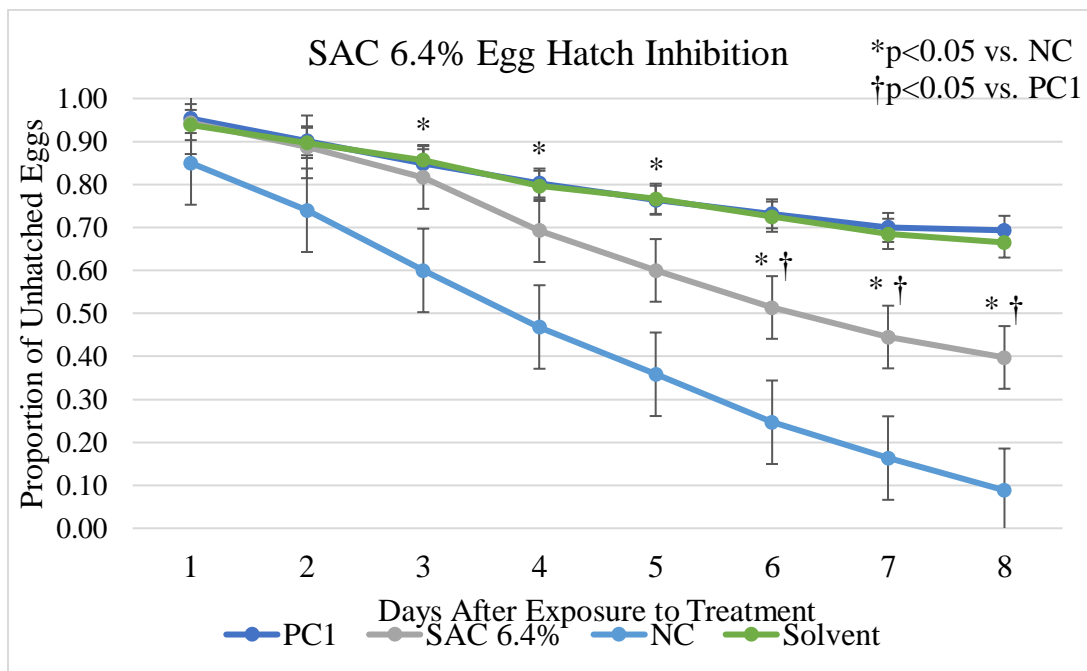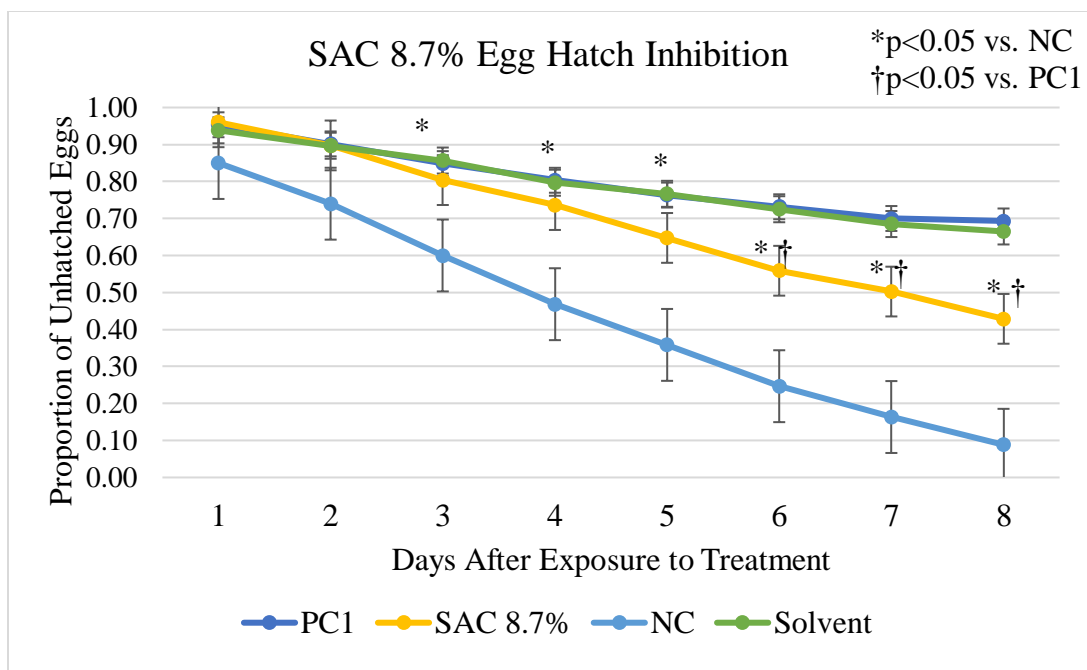**Figure 12.** Effect of SAC 8.7% concentration on *H. contortus* egg hatch inhibition over 8-day experimental window. *p<0.05 vs. NC; †p<0.05 vs. PC1.

VII. DISCUSSION

While none of the three plant extracts exhibited as strong of an ovicidal effect when compared to the Ivomec used for the PC1, they demonstrated some degree of ovicidal capabilities. This suggests that the three extracts are a better treatment option than providing no treatment option at all to goats infected with *H. contortus.* Providing producers with practical, immediate solutions to mitigating anthelmintic-resistant gastrointestinal nematodes is a more viable, alternative option compared to lengthy and costly novel anthelmintic development (McKellar 1994; Witty 1999; Waller 2006). Given that allicin is highly unstable when exposed to blood or changes in temperature or pH (Lawson et al. 1992; Lawson and Wang 1993; Lawson 1998; Lawson and Hunsaker 2018), it is unlikely that allicin was responsible for exhibiting the strongest ovicidal performance when compared to PSO and SAC. Rather, it is more plausible that allicin quickly degraded into various secondary compounds which were mostly responsible for the stronger ovicidal performance (Agarwal 1996; Rosen et al. 2001). This does not exclude allicin as a future anthelmintic treatment, rather an optimal delivery system that could ensure that allicin reaches its intended target within a ruminant stomach might be a suitable research endeavor. The rumen, the largest chamber of the ruminant four-chamber stomach, acts as the main site of rumination and fermentation, and may make it difficult for directly enacting the medicinal benefits of certain anthelmintics (LaCount et al. 1996; Cao et al. 2008). This might explain why plasma concentrations, persistence, and availability of anthelmintics deemed therapeutic for other ruminants like sheep and cattle, are significantly lower in goats, making the determination of a therapeutic dose of synthetic anthelmintics for goats more difficult (Aksit et al. 2015; Myers et al. 2020).

Several approaches have been formulated to bypass the rumen. Researchers have focused on simple heat or chemical treatments of proteins, utilization of low-soluble peptides or amino acid analogues, and the use of lipids as a protective matrix for various proteins (Sklan and Tinsky 1993; Wu and Papas, 1997; Rossi et al. 1999; Yoshimaru et al. 1999; Ranaweera et al. 2020). Unfortunately, many of these rumen bypass delivery systems exhibited variable responses after encountering the neutral pH of the rumen, its microflora, and digestive components found within the rumen. This occasionally caused disruption in the release of dietary or medicinal supplements in the abomasum (Cao et al. 2008). Determining an effective rumen bypass system of delivery that could allow allicin to reach the abomasum before its quick metabolization might increase its effectiveness as an anthelmintic alternative. By doing so, SAC and various other secondary metabolites might also reach the abomasum, targeting *H. contortus* worms directly. Given the minimal ovicidal results exhibited by PSO, it might also benefit from a rumen bypass system of delivery to ensure it reaches the abomasum. However, certain secondary compounds of garlic and pumpkin seed oil might be able to survive the ruminal fermentation process and reach the abomasum relatively quickly. Garlic and pumpkin seed oil are known to contain various phenolic compounds, which have been reported to bypass the rumen microbes quite readily, exhibiting a recovery rate of 60% after ruminal fermentation has occurred (Oh et al. 2013; Peiretti et al. 2017). Oh et al. (2013) also reported that liquid components exit the rumen at a rate of 20%/hr. Altering the pH of the abomasum might prove beneficial as well through the utilization of herbal compounds. The abomasum pH directly influences the egg laying capabilities of *H. contortus* (Mravčáková et al. 2020). Honde and Bueno (1982) reported an acidic pH in the

abomasum lower than five can increase the volume of egg production by 106%. A small increase in the pH of the abomasum can theoretically diminish egg production, which might be further compromised if these secondary compounds of garlic and pumpkin seed oil successfully reach the abomasum.

VIII. CONCLUSION

When assessing the ovicidal capabilities of allicin, there are definite implications that allicin might be a viable treatment when used as an ovicidal agent. There is an intermediary effect exhibited when comparing the 1% allicin treatment to both the NC and PC1 on days seven and eight. The 2% allicin treatment also exhibited an intermediary effect when compared to both the NC and PC1 on day eight. Although an intermediary effect was not exhibited by the 4% allicin treatment, it is worth noting that the significant effect of this treatment compared to the NC that began only after day four strengthens the argument that allicin might be an effective ovicidal agent. When looking at SAC, this idea of an intermediary effect is apparent at all three concentrations beginning on day six, suggesting that the secondary metabolites of allicin are mostly responsible for its ovicidal capabilities. Although the PSO only showed a significant effect when compared to the PC1, the three different treatment concentrations of PSO still outperformed the NC, suggesting partial ovicidal capabilities. These plant extracts may offer immediate, practical solutions to producers dealing with anthelmintic-resistant *H. contortus* infections within their goat herds. These extracts could also be easily incorporated into current treatment regimens without entirely eliminating synthetic anthelmintic reliance. Rather, these plant extracts might offer synergistic benefits when used with synthetic anthelmintic treatments to increase ovicidal efficacy.

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