Assessment of nematocidal activity of novel N-alkyl analogues of nitrofurantoin on *Caenorhabditis elegans* nematode model

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ABSTRACT

Caenorhabditis elegans is considered as a simple but important invertebrate model which provides a versatile platform of various experiments such as drug studies. The *C. elegans* is commonly used in anthelmintic drug studies because it shares common anatomical and physiological characteristics with majority of species in the phylum Nemathelminthes. Amongst nematodes infecting livestock, *Haemonchus* spp., *Trichostrongylus* spp. and *Paramphistomum* spp. commonly infect small stock and result in devastating effects, both clinically and economically. Several of pharmaceutical chemicals used to manage these gastrointestinal nematodes are beginning to lose their effectiveness due to resistance in these nematodes. Alternative drugs that can possibly be used in the successful management of small ruminants are nitrofurantoin derivatives. The aim of this study was to assess the effect of novel N-alkyl analogues of nitrofurantoin on the larval development and larval mortality on *C. elegans* nematode model.*C. elegans* eggs were conserved and maintained on agar plates with *E. coli* as a food source. After an incubation period of three days, worms with the same life stage were obtained by means of following the ISO standard for water quality that enables the determination of turbidity. Ten L1 stage larvae were placed in a 12 well plate as well as a food medium, M9-medium and 150 µl of the eight different nitrofurantoin derivatives (that were dissolved in a 10% DSM/PBS solution). The wells with the L1 stage larvae were then incubated for four days at 21 °C. After the incubation period, the test was terminated and the nematodes were transferred to a petri dish for observation.It is clear that compounds 104, 105, and 106, had the best nematocidal effect on the round worms, with compound 106 being the most effective at killing *C. elegans.* Compounds 104, 105, and 106, had the least amount of larval development, with compound 105 inhibiting larval development the best. Compound 104 had the least amount of L_1 stage larvae and the highest number of dead larvae. This means that compound 104 caused *C. elegans* to reproduce less offspring than the other compounds.

This study has shown that derivatives of N-alkyl analogues of nitrofurantoin are all unique have *in vitro* anthelminthic activity against *C. elegans* nematode model. These findings pave way for future studies that should confirm efficacy in selected nematodes that infect livestock such as *Haemonchus contortus* and then conduct *in vivo* efficacy and toxicity tests on experimental animals.

Key words: *Caenorhabditis elegans,* Nematoda, nitrofurantoin, derivatives

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CHAPTER 1 INTRODUCTION AND LITERATURE REVIEW

1.1 Economic Importance of Small Stock

Sheep and goats are vital for our survival, because they provide a variety of food sources for human beings, from milk to meat (Iyiola-Tunji, 2015). Small ruminants are not only used for food consumption, they also provide us with wool, hair, skin and manure (Iyiola-Tunji, 2015; Zach *et al.*, 2012). These fibrous substances are used in fabric production companies (Iyiola-Tunji, 2015; Zach *et al.*, 2012). The wool of sheep is used for the production of clothes whereas the skin and by-products of slaughter for shoes and various of leather products (Iyiola-Tunji, 2015). Manure of small ruminants can be of some agricultural use as well (Iyiola-Tunji, 2015). The manure helps to enhance the soil quality, by adding important nutrients (Amanullah *et al.*, 2019; Irfan Sohail *et al.*, 2019; Iyiola-Tunji, 2015).

Sheep can be converted to cash very quickly when sold. In South Africa, sheep contribute 10% of animals product income (Cloete & Olivier, 2010; Lubabalo, 2016; Schoeman *et al.*, 2010). The bigger part of the percentage is due to sheep meat (somewhat 60.6% of the 10%), and wool (31.4%) (Cloete & Olivier, 2010). Thus small ruminants of South Africa produce a –ZAR 3 billion turn over (Cloete & Olivier, 2010).

1.2 Nematoda

Nematoda is a phylum which describes a group of animals, round worms, with indistinguishable body structuring with characteristics of a lengthened, lean and unsegmented body as illustrated in Figure 1 (Okkema, 2017; Thorp *et al.*, 2010). Organisms that represent the phylum Nematoda can act as bio-indicators to water

quality and changes to the environment (Decraemer *et al.*, 2019). There is an estimation that round worms (Mayer & Donnelly, 2013) have been alive for one billion years (Lambert & Bekal, 2002). Nematodes occur in a wide collection of habitats which represents various water bodies, soil, plants and parasitize animal species from reptiles, wild animals to domesticated animals and livestock (Colville & Berryhill, 2007; Decraemer *et al.*, 2019; Mayer & Donnelly, 2013; Nielsen *et al.*, 2014; Tattar, 1989; Thorp *et al.*, 2010).

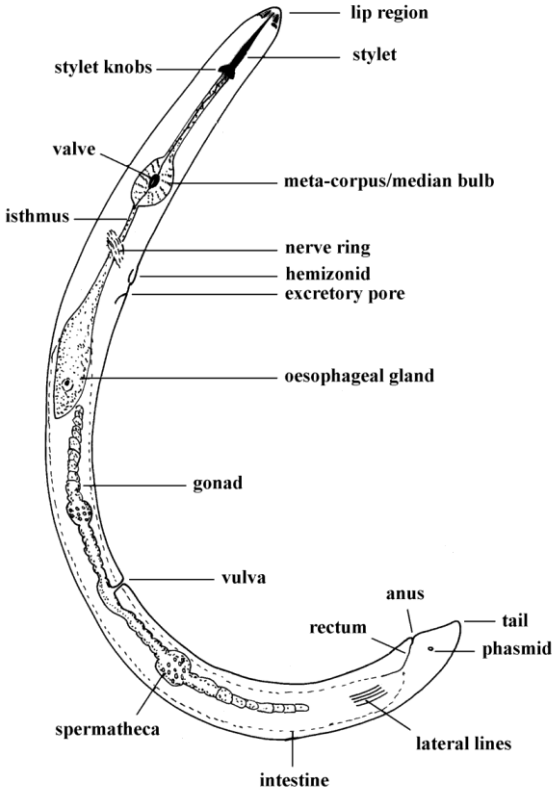


Figure 1: General morphological features of nematodes (Stirling & Nicol, 2002).

Nematodes that infect and cause illness in mammals can be put into two groups, namely, ambushers and cruisers (Gang & Hallem, 2016). Ambushers are nematodes that stay stationary, meaning these nematodes wait for their host instead of searching for it (Gang & Hallem, 2016). An example of both ambushers and

cruisers are entomopathogenic nematodes (EPNs) (Gang & Hallem, 2016). EPNs are nematodes that are beneficial to the environment because they infect and destroy a diverse group of insect pests (Gang & Hallem, 2016). These nematodes are wildly used around the world as a biological control method (Gang & Hallem, 2016). EPNs ambush hosts by remaining completely stationary thereby latching on when a host passes by like *Steinernema carpocapsae* (Gang & Hallem, 2016; Gaugler *et al.*, 1997). A contradiction is observed with the cruiser group, these nematodes appear to spend more of their time crawling in search of their host (Gang & Hallem, 2016). An example of cruiser nematodes are the infective juveniles of EPNs which searches for their host like *Steinernema glaseri* (Gang & Hallem, 2016; Gaugler *et al.*, 1997). Parasitic nematodes use different methods to detect their host (Chaisson & Hallem, 2012; Gang & Hallem, 2016). One of these methods is host-emitted chemosensory cues (Gang *et al.*, 2020; Gang & Hallem, 2016). Host-emitted chemosensory cues is the process where parasitic nematodes use CO_2 to locate their host (Gang & Hallem, 2016).

In South Africa small ruminants are commonly infected by *Haemonchus* spp., *Trichostrongylus* spp. and *Paramphistomum* spp. (Tsotetsi *et al*., 2003) resulting in both clinical and economical effects. The *Haemonchus contortus* nematode stand out as the most problematic and economically important gastrointestinal parasite infecting small stock, particularly sheep (Mafuna *et al.,* 2021).

1.3 Biology of Haemonchus contortus

The biology of *H. contortus* is much like any other roundworm which can be described as a multicellular organisms that contains 1000 cells or less with bodies that are transparent, which makes their anatomy uncomplicated (Lambert & Bekal, 2002). The body of the roundworm is lean and slender and represents a threadlike form (Kuchai *et al.*, 2012; Widiarso *et al.*, 2018). The anterior part of the worm's body is more broad and flattened in contrast to the posterior part (Kuchai *et al.*, 2012). The buccal cavity is considered tiny with a transparent tooth that stick out of the dorsal wall (Kuchai *et al.*, 2012).

Infantile worms develop into adults in the abomasum whilst sucking the blood of their host (Delano *et al.*, 2002; Gelberg, 2017). The parasite is able to do this by means of attaching itself to the lining of the membrane, cutting the membrane with a sharp organ located inside the mouth (Delano *et al.*, 2002; Gelberg, 2017). When the worms are done sucking the blood of its host, it will free itself via detachment, resulting in the host bleeding for several minutes consequently resulting in blood loss from the sheep (Delano *et al.*, 2002; Gelberg, 2017).

The free-living stage consist out of the eggs, first-stage larvae (L_1), second stage larvae (L_2), third stage larvae (L_3) and the fourth stage larvae (L_4), exposed to the harsh conditions of the environment, such as dramatic temperature and humidity changes, which will affect the population (Gasser & von Samson-Himmelstjerna, 2016).

The life cycle of *H. contortus* ranges between 17 to 21 days and is illustrated below in Figure 2 (Simpson, 2015). The life cycle starts with the larvae, which is considered the infective stage, and is ingested by sheep while grazing (Roeber *et al.*, 2013a)

and is transported to the abomasum for further development (Roeber *et al.*, 2013a; Simpson, 2015). Females can produce up to 5,000 to 10,000 eggs per day which are spread through the feces of small ruminants (Miller, 2010; Simpson, 2015). The eggs develop into larvae only when conditions are favorable, and move into the grazing area where sheep and goats digest the larvae (Miller, 2010; Roeber *et al.*, 2013a; Simpson, 2015; Yang *et al.*, 2017).

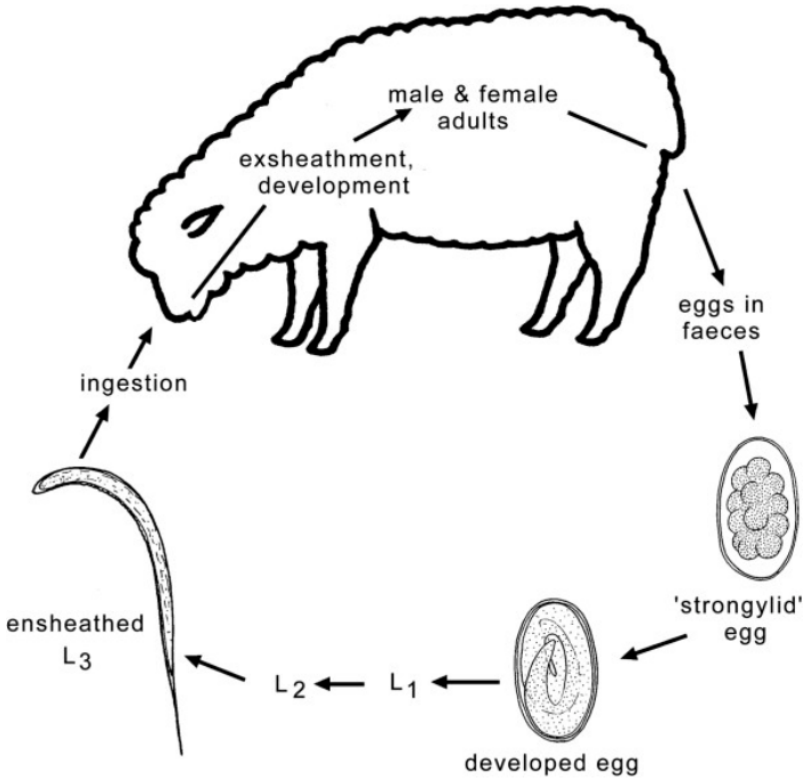


Figure 2: Life cycle of *H. contortus* (Besier, 1987; Roeber *et al.*, 2013b).

Even though *H. contortus* usually needs warm and wet temperatures to develop correctly, it is noted that it has outstandingly adaptable characteristics, and can therefore be found in geographical regions that are cold (Besier *et al.*, 2016; Gasser & von Samson-Himmelstjerna, 2016). Another reason these roundworms can be found in colder geographical regions is due to climate change, causing the earth's temperature to rise all over the globe, thus supporting the environmental conditions

it needs to survive (Besier *et al.*, 2016). The eggs and larvae of *H. contortus* can be found in the winter months of the year, in contrast to the adults which is found inside the host when the temperature rises (Gasser & von Samson-Himmelstjerna, 2016).

1.4 Haemonchosis

H. contortus causes haemonchosis in sheep (Besier *et al.*, 2016b; Constable *et al.*, 2017; Roeber *et al.*, 2013a; Uzal *et al.*, 2016), and can be described as the most dangerous nematode out of all common nematodes (Gasser & von Samson-Himmelstjerna, 2016). Haemonchosis is a parasitic disease that affects livestock worldwide with signs and symptoms including anemia, weight loss, reduced growth, production loss and mortality (Besier *et al.*, 2016b; Martin, 1997; Roeber *et al.*, 2013; Suleiman, 2017; Widiarso *et al.*, 2018; Ismail *et al.*, 2020). Blood loss is the main symptom in small ruminants and is an indicator of the beginning of the L4 larval development (Butler, 1966; Gasser & von Samson-Himmelstjerna, 2016). The *H. contortus* adults can suck more than 45 µl blood per day (Gasser & von Samson-Himmelstjerna, 2016). The level of seriousness of the infection is related to the amount of worms in the host (Gasser & von Samson-Himmelstjerna, 2016). Some of the first warning signs of infections is when certain sheep within the flock get tired faster than the rest. Upon examination of these individuals, their eyes and mouth membranes as well as their skin would appear to be paler, compared to a healthy sheep (Butler, 1966; Suleiman, 2017).

The *H. contortus* infections are categorized into three stages namely, hyperacute, acute and chronic (Gasser & von Samson-Himmelstjerna, 2016; Selemon, 2018). During the hyperacute stage it is possible for the host to die within a week without any clinical signs or symptoms of the initial infection. However, this stage is rare. When the host develops acute haemonchosis, it is common for the that animal to also develop severe anemia and edema bottle jaw over a prolonged period, and it is possible that it may die within a few weeks (Besier *et al.*, 2016c; Simpson, 2015).

The chronic stage of infection is referred to as a natural infection and can easily be unnoticed (Gasser & von Samson-Himmelstjerna, 2016; Selemon, 2018)

1.5 Management of Haemonchus contortus

Diagnosis

The diagnosis of *H. contortus* goes hand in hand with the control of this nematode (Gasser & von Samson-Himmelstjerna, 2016; Höglund *et al.*, 2019). When haemonchosis in livestock is suspected it is important to have an accurate diagnosis, because this parasite has the potential to cause great amount of damage (Gasser & von Samson-Himmelstjerna, 2016). Clinical diagnosis on the other hand is based not only on clinical evidence such as signs and symptoms the host displays, but also faecal egg count results (Höglund *et al.*, 2019; Miller, 2010). . The faecal worm egg count (FWECs) is a vital technique in the diagnosis of haemonchosis and contribute to the integrated pest management (IPM) aspect of the parasite (Gasser & von Samson-Himmelstjerna, 2016). IMP strategies include the reproduction of livestock with genetic characteristics such as natural resistance to nematodes (Gasser & von Samson-Himmelstjerna, 2016).

1.6 Caenorhabditis elegans

Introduction to Caenorhabditis elegans

The *Caenorhabditis elegans* was first described in 1897 by a French scientist named Emile Maupas (El-Bawab, 2020; Riddle *et al.*, 1997). It is a free-living, small nematode with a worldwide distribution, and belongs to the phylum Nematoda (Altun & Hall, 2006; Corsi *et al.*, 2015). *Caenorhabditis elegans* is not just a soil nematode but can be found in rotting organic materials which contains enough bacteria as a food source (Altun & Hall, 2006; Corsi *et al.*, 2015).

Caenorhabditis elegans is considered as a simple but important invertebrate model which provides a versatile platform for various experiments such as drug studies (Connor & Watts, 2019; Hahnel *et al.*, 2020; Muschiol *et al.*, 2009; Rabinowitch & Schafer, 2017; Ro & Rosenzweig, 2018). *C. elegans* contain multiple advantages for such studies because of their short life cycle, small sizes, large number of offspring, their simplicity of cultivation, which is maintenance-free , just to name a few (Artal-Sanz *et al.*, 2006; Carranza-García & Navarro, 2020; Corsi *et al.*, 2015). *Caenorhabditis elegans* is used in various of studies to represent the whole phylum Nematoda, because they share the main characteristics of the nematode body plan (Geary & Thompson, 2001; Gilleard, 2004; Hahnel *et al.*, 2020; Holden-Dye & Walker, 2014; Riddle *et al.*, 1997; Salinas & Risi, 2018). This species of nematode is easily grown and maintained on agar plates containing *Escherichia coli* as the primary food source (Altun & Hall, 2006; Artal-Sanz *et al.*, 2006; Corsi *et al.*, 2015). Even tough *C. elegans* is so simple, it does exhibit a wide range of behavioral properties from foraging to sensory responses to also having the ability to learn from its environment (Altun & Hall, 2006; de Bono, 2003; Rankin, 2002).

Caenorhabditis elegans stays true to the common nematode body structure consisting of unsegmented, cylindrical body that narrows at the ends, accompanied by outer tube as well as the inner tube that is separated by the pseudocoelom space (Altun & Hall, 2006). When the genomic structure of *C. elegans* was compared to that of *H. contortus* is was revealed that *H. contortus* shares around 70% of their predicted genes with *C. elegans* (Berriman *et al.*, 2019; Hahnel *et al.*, 2020). Therefor the use of *C. elegans* is supported in anthelmintic drug research (Hahnel *et al.*, 2020)

Life cycle of Caenorhabditis elegans

The life cycle of most nematodes consists of an embryonic stage, followed by four larval stages (L_1, L_2, L_3, L_4) and then finally into an adult that can reproduce (Altun & Hall, 2006; Byerly *et al.*, 1976; Carranza-García & Navarro, 2020; Palikaras & Tavernarakis, 2013). *Caenorhabditis elegans* life cycle is at its most optimal at 25℃ where it develops from an egg into a reproductive adults in just three days which is considered a quick life cycle as illustrated in Figure 3 (Altun & Hall, 2006; Artal-Sanz *et al.*, 2006; Carranza-García & Navarro, 2020; Corsi *et al.*, 2015; Muschiol *et al.*, 2009). Embryogenesis within this species takes roughly 16 hours at 20℃ to 25℃ (Corsi *et al.*, 2015; Palikaras & Tavernarakis, 2013). This nematode primarily reproduces through self-fertilizing as most individuals are hermaphrodites, although males do occur within the population (Carranza-García & Navarro, 2020; Corsi *et al.*, 2015; Palikaras & Tavernarakis, 2013). Hatched larvae are the length of 0.25 mm, whereas the adult worms can grow up to 1 mm long (Artal-Sanz *et al.*, 2006; Breed, 2017; Corsi *et al.*, 2015; Riddle *et al.*, 1997).

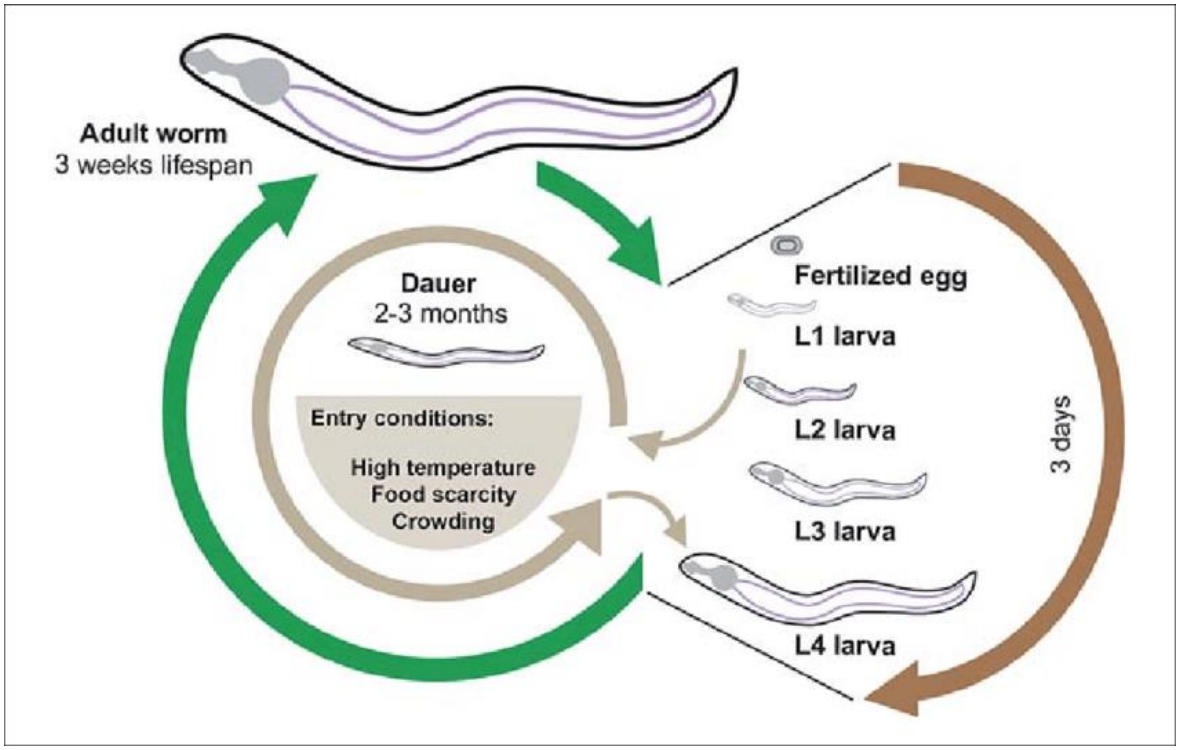


Figure 3: The life cycle of *C. elegans* (Ewald *et al.*, 2017).

1.7 Anthelmintics

Background

Anthelmintics are described as a medicinal factor that is used in the elimination of parasitic nematodes infecting hosts (Al-Hadiya, 2010). Gastrointestinal nematode (GINs) infections of livestock have been treated by mostly pharmaceutically derived anthelmintics (both synthetic and semi-synthetic drugs) for more than three decades (Ihler, 2010; Mphahlele *et al.*, 2019; Suleiman, 2017). Anthelmintics can be interpreted as substances that causes the amount of parasites in the host to decrease to the degree that the host can endure it (Suleiman, 2017). These substances reduce the parasites in the host by causing death to the parasite, referred to as a vermicide (Mphahlele *et al.*, 2019; Suleiman, 2017). Anthelmintics can also delay the growth of nematodes which is known as vermifuge, or by means of penalization (Mphahlele *et al.*, 2019; Suleiman, 2017).

Broad spectrum drugs to be used against nematode parasites were documented in the year 1950s-1960s, they were phenothiazine and piperazine (Holden-Dye & Walker, 2014; Suleiman, 2017). The next generation of a broad spectrum drug for parasitic nematodes included benzimidazoles, probenzimidazoles, imidazothiazoles as well as tetrahydropyrimidines (Suleiman, 2017).

Various pharmaceutical chemicals used to manage these gastrointestinal nematodes are beginning to lose their effectiveness due to resistance (Baltrušis *et al.*, 2018; Craig, 1993; Kotze & Prichard, 2016; Mphahlele *et al.*, 2019). Anthelmintic resistance is described as the capacity of parasites to withstand drug doses that would typically kill parasites of the same species and stage (Mphahlele *et al.*, 2021)

Evolution has caused *H. contortus* to have a tendency in developing resistance in anthelmintic drugs, even when the drugs have only been used in a short period of time (few years) (Gasser & von Samson-Himmelstjerna, 2016; Martin, 1997; Mphahlele *et al.*, 2019). The best explanation for this phenomenon is due to parasite's high genetic diversity within the large population sizes (Gasser & von Samson-Himmelstjerna, 2016). The same level of resistance in *H. contortus* can be observed in populations that have more or less the same amount of genetic diversity (Gasser & von Samson-Himmelstjerna, 2016). In other words, the higher the genetic diversity within a population, the higher potential it has to evolve in becoming resistant (Gasser & von Samson-Himmelstjerna, 2016).

There are three known mutations on the isotype-1 β-tubulin gene referred to as the F200Y (T<u>A</u>C), E198A (G<u>C</u>A) AND F167Y (T<u>A</u>C) that make it possible to develop resistance (Gasser & von Samson-Himmelstjerna, 2016). The mutation that is the most frequent is the F200Y (T<u>A</u>C), with the E198A (G<u>C</u>A) mutation which is scarce (Gasser & von Samson-Himmelstjerna, 2016).

One of the most common drugs used on gastrointestinal nematodes is the benzimidazole group (Appusamy, 2015; Baltrušis *et al.*, 2018; Barrere *et al.*, 2013; Kotze & Prichard, 2016; Van Wyk *et al.*, 1997). Anthelmintic resistance has been reported and poses a serious problem throughout the world (Fissiha & Kinde, 2021). The very first case of resistance in *H. contortus* to benzimidazole anthelmintics in South Africa was described in 1975 (Bakunzi, 2003; Mphahlele *et al.*, 2021; Van Wyk *et al.*, 1997, 1999).

Mode of action of anthelmintic drugs

The ideal anthelmintic drug has to consist out of a wide spectrum of activity against various of parasitic stages as described in Table 1.1 below (Martin, 1997; Suleiman, 2017). When treating livestock with anthelmintic drugs it is important to remember that these drugs need to have selective toxic traits on a nematode parasite (Appusamy, 2015; Martin, 1997). These drugs accomplishes this task by either obstruction of the metabolic processes that are vital to the nematode (but do not interfere with the processes of the host), or by properties of the drug that causes the parasite to be exposed to higher concentrations of the drug than the host as illustrated by Table 1.1 below (Abongwa *et al.*, 2017; Appusamy, 2015; Martin, 1997). When resistance in these nematodes occur with a specific drug, it is expected that other drugs with the same mode of action will not work, whereas drugs with a different mode of action will likely be affective (Martin, 1997).

Biochemical mechanisms of resistance

Resistance in gastrointestinal nematodes are a serious situation, thus the biochemical mechanisms of resistance should be researched and understood, only then can resistance in these worms bemonitored (Gasser & von Samson-Himmelstjerna, 2016; Guo *et al.*, 2016). The mechanisms responsible for resistance can be summarized as the following points, 1) when the molecule target site changes, resulting in a weaker interaction with the drug to the target site; 2) the metabolism changes of the nematode, which can result in complete removal of the drug or non-functioning of the drug by means of inactivation; 3) the distribution of the drug changes, thereby causing the drug to never reach its target site; 4) amplification of the molecular target gene to avoid drug action; and 5) the increase

of molecular target by means of extra proteins that are closely related and are not affected by the drug (Gasser & von Samson-Himmelstjerna, 2016; Köhler, 2001; Rew, 1978).

Anthelmintic resistance management

It is widely known that the use of the same anthelmintic group, incorrect dosage measurements, prophylactic mass treatment, as well as the frequent use of the same drug in the control of parasitic nematode contributes to parasitic nematodes to develop resistance (Shalaby, 2013). Firstly, underdosing is one of the main factors which can develop resistance (Fissiha & Kinde, 2021; Gasser & von Samson-Himmelstjerna, 2016). This problem seems very common, either because the individual lacks the consequential knowledge of proper dosage, with the miscalculations of the weight of the animals (Gasser & von Samson-Himmelstjerna, 2016).

Resistance management in parasitic nematodes can also be done with non-chemical method such as good management, quality pastures, rotation of pastures, grazing among different animal species host animals that are well nourished selective breeding of the host as well as biological control (Gasser & von Samson-Himmelstjerna, 2016; Kelly *et al.*, 2010; Maqbool *et al.*, 2017).

Anthelmintic combination

Combinations of different anthelmintics with the same mode of action is commonly used, and is available in most countries (Bartram *et al.*, 2012). The reasons these anthelmintics are used in combinations is to ensure that the anthelmintics are

successful, and to prolong the process of the nematodes to develop resistance (Bartram *et al.*, 2012; Graef *et al.*, 2013; Leathwick *et al.*, 2015).

Table 1-1: Different modes of action of anthelmintic drugs (Lecova *et al.*, 2014; Martin, 1997; Mukherjee *et al.*, 2015; Wolstenholme & Rogers, 2005).

Anthelmintic name	Target site/mode of action	Helminths	References
Benzimidazoles (Albendazole, cambendazole, oxibendazole, febendazole, flubendazole, mebendazole, thiabendazole, netobimine)	Targets the microtubules of the cytoskeleton. Transport of the secretory granules are blocked as well as other sub-cellular organelles	*Ascaris lumbriciodes* *Ancylostoma duodenale* *Enterobius vermicuclaris* *Trichinella spiralis*	(Holden-Dye & Walker, 2014; Lecova *et al.*, 2014; Martin, 1997)
Clorsulon	Inhibits phosphoglycerate kinase and mutase	Liver flukes	(Martin, 1997; Mukherjee *et al.*, 2015)
Diamphenethide	Inhibitions of malate metabolism	*Fusciola hepatica*	(Martin, 1997)
Diethylcarbamazine citrate	Inhibits arachidonic acid metabolism as well as the stimulation of innate immunity	*Wucheraria bancrofti* *Burgia malayi* *Dirofilaria immits*	(Martin, 1997; Mukherjee *et al.*, 2015)
Haloxon dichlorvos	Acetylcholinesterase inhibition	*Ancylostoma caninum*	(Martin, 1997; Mukherjee *et al.*, 2015)

Ivermectin, Abamectin, Doramectin, Moxidectin, Milbemycin D	GluCl potentiators, ion channels	*Oncocerca volvulus* *Onocerca loaloa*	(Holden-Dye & Walker, 2014; Lecova *et al.*, 2014; Mukherjee *et al.*, 2015)
Piperazine	(GABA) Gamma-aminobutyric acid agonist	Intestinal roundworms- *Ascaris lumbricoides* Threadworm- *Enterobius, Oxyuris, Vermicularis*	(Holden-Dye & Walker, 2014; Martin, 1997)
Praziquantel	Increases the permeability towards Calcium results in increase calcium influx leading to muscular contracture	*Schistostoma hematobium* *Schistostoma mansoni* *Schistosoma japonicum* *Tenia saginata* *Tenia solium* *Diphyllobothrium latum* *Hymenolepsis nana*	(Martin, 1997; Mukherjee *et al.*, 2015)
Salicylanilides, Rafoxanide, Oxyclozanide, Brotianide, Nitroxynil, Niclopholan, Hexachlorophene, Dibromsalan, Chrorinated sulfonamides	Bioenergenetics, Proton ionophores	Gastrointestinal roundworms- *Bunostonum* spp, *Haemonchus* spp, *Oesophagostomum* spp	(Lecova *et al.*, 2014; Martin, 1997; Mukherjee *et al.*, 2015)

		Liver fluke- *Fasciola hepatica* Tapeworms- *Taenia* spp*, Moniezia* spp	
Tetrahydropyrimidines, Imidazothiazoles, Levamisole, Butamisole, Bephenium, Thenium, Methyridine	Nicotinic agonist	Most gastrointestinal nematodes of sheep, cattle, horses, dogs, cats, and pigs. *Ancylostoma caninum* *Ascaris lumbricoides*	(Holden-Dye & Walker, 2014; Lecova *et al.*, 2014; Mukherjee *et al.*, 2015)

1.8 Anthelmintic groups

Amino-acetonitrile derivatives (AADs)

Amino-acetonitrile possess a wide spectrum of activity against parasitic nematodes that show signs of resistance towards benzimidazoles, imidazothiazoles as well as macrocyclic lactones (Abongwa *et al.*, 2017; Williamson, 2014). Monepantel is the first member of AADs to be developed for the use of nematodes in sheep and goats (Gasser & von Samson-Himmelstjerna, 2016). Monepantel was shown to have a low to zero toxicity level to sheep and goats and other livestock (Keiser & Utzinger, 2010), and it is known to have an eccentric mode of action against the nicotinic acetylcholine receptors in the parasite (Gasser & von Samson-Himmelstjerna, 2016). A study done on sheep in Uruguay reported two farms treated with monepantel exhibited resistance to AADs as the fecal egg count did not decrease with treatment (Brom *et al.*, 2015; Mederos *et al.*, 2014).

Benzimidazoles

Benzimidazoles are one of the first ever classes of broad-spectrum anthelmintics to be used to treat livestock against parasitic nematodes (Abongwa *et al.*, 2017; Gasser & von Samson-Himmelstjerna, 2016; Mphahlele *et al.*, 2019). This group affect nematodes on cell level, by means of hindering the polymerization of microtubules, which causes cell death (Gasser & von Samson-Himmelstjerna, 2016; Vanden Bossche, 1990). However, the overuse of benzimidazole has resulted in loss of its clinical effectiveness which has led to nematodes resistance against it worldwide (Abongwa *et al.*, 2017; Mphahlele *et al.*, 2019; Vanden Bossche, 1990).

Organophosphates

Organophosphates are derived from phosphoric and/or phosphoric acid (Abdelsalam, 1987; Gupta, 2014). Organophosphates can be used in several ways including insecticides, fungicides, herbicides and nematicides (Ganie *et al.*, 2022). These anthelmintics act by hindering the acetyl-cholinesterase activity, hence there is a possibility that they may be toxic to the mammalian host as well (Ganie *et al.*, 2022; Gasser & von Samson-Himmelstjerna, 2016; Gupta, 2014).

Macrocyclic lactones

These anthelmintics belong to drugs produced through fermentation by soil microorganisms of the genus *Streptomyces* (Abongwa *et al.*, 2017; Vercruysse & Claerebout, 2014a). Macrocyclic lactones include different groups like ivermectin, selamectin, moxidectin, and milbemycin oxime (Abongwa *et al.*, 2017; Wolstenholme *et al.*, 2016). Ivermectin, developed in the 1980's (Abongwa *et al.*, 2017; Gasser & von Samson-Himmelstjerna, 2016) and is the only member of this group that has been found suitable for human consumption (Wolstenholme *et al.*, 2016). Macrocyclic lactones act by stopping transmission in the parasite nervous system by means of increasing the glutamate-gated chloride channels (Abongwa *et al.*, 2017; Gasser & von Samson-Himmelstjerna, 2016).

Salicylanilides and substituted phenols.

Members of this group work only on adult stages of the parasite (Vercruysse & Claerebout, 2014b). Salicylanilides and substituted phenols work via hindering the energy metabolism (Gasser & von Samson-Himmelstjerna, 2016).

Spiroindoles

The initial member of the Spiroindoles is derquantel (Gasser & von Samson-Himmelstjerna, 2016; Vercruysse & Claerebout, 2014c). Derquantel is combined with abametin (Vercruysse & Claerebout, 2014c), as derquantel is not effective on its own at controlling parasitic nematodes (Gasser & von Samson-Himmelstjerna, 2016).

Tetrahydropyrimidines/Imidazothiazoles

These compounds are known to have a broad-spectrum anthelmintic activity against nematodes, but it has no effect on cestodes or trematodes (Holden-Dye & Walker, 2014).

Levamisole

Levamisole is an anthelmintic that affects the nervous system of parasitic nematodes (Vercruysse & Claerebout, 2014d). This anthelmintic like tetrahydropyrimidines is a broad spectrum drug that is easy to administer (Gasser & von Samson-Himmelstjerna, 2016; Vercruysse & Claerebout, 2014).

1.9 Specific-and-nonspecific resistance

It has been identified with previous studies that *H. contortus* shows resistance to both benzimidazole and levamisole (Baltrušis *et al.*, 2018; Gasser & von Samson-Himmelstjerna, 2016; Shokrani *et al.*, 2012). The target site for benzimidazole is β-tubulin (Baltrušis *et al.*, 2018; Gasser & von Samson-Himmelstjerna, 2016; Lubega & Prichard, 1990; Shokrani *et al.*, 2012). Resistance was observed through specific changes that occurred in the β-tubulin gene sequence, this process is known as specific resistance (Gasser & von Samson-Himmelstjerna, 2016). Round worms

that are susceptible to die from benzimidazole show phenylalanine at the β-tubulin site, whereas worms that develop resistance show tyrosine, the result being a single nucleotide polymorphism (Gasser & von Samson-Himmelstjerna, 2016).

The resistance of *H. contortus* to the drug levamisole has been identified by the short cutting of the UNC-63 gene (Gasser & von Samson-Himmelstjerna, 2016). Levamisole causes the body wall muscles of nematodes to hyper contract resulting in death (Culetto *et al.*, 2004). Resistance to levamisole has been observed in 12 genes including UNC-38, UNC-29, UNC-63 and LEV-1 (Culetto *et al.*, 2004). These genes encode nicotinic acetylcholine receptor (nAChR) subunits (Culetto *et al.*, 2004). It is debated that the resistance occurrence can possibly happen due to natural conditions (production of levamisole resistant phenotypes) (Gasser & von Samson-Himmelstjerna, 2016).

Like many animals, parasitic nematodes have numerous important enzymes and proteins that are responsible for transporting as well as protecting the nematode against foreign substances (toxins) (Gasser & von Samson-Himmelstjerna, 2016). This mechanism is known as nonspecific resistance mechanism and possess three detoxification processes, namely excretion, modification and conjugation of foreign substances (Gasser & von Samson-Himmelstjerna, 2016; Ramos *et al.*, 2018). Currently, it is only assumed that these three detoxification possesses are also involved in the detoxification of anthelmintic drugs (Gasser & von Samson-Himmelstjerna, 2016).

1.10 Alternative drug

Nitrofurantoin

Nitrofurantoin and metronidazole are part of the nitroheterocyclic drugs which are commonly used for infectious diseases (Hof, 1988; Vardanyan & Hruby, 2006). Nitrofurantoin has shown to have a powerful antimicrobial effect, especially on various of bacterial groups, protozoa and worms (Hof, 1988; Sharma & Anand, 1997). This drug is used for several treatments in humans including urinary tract infections (pyelitis, pyelonephritis, cystitis, urethritis) as well as treating certain cancers (Howard, 2007; Thakuria *et al.*, 2017; Vardanyan & Hruby, 2006; Zuma *et al.*, 2019).

Even though nitrofurantoin is mainly used for its antibacterial activity, it has been noted that nitrofurantoin has been able to kill microfilariae and the adult worms of *Litomosoides carinii* (Sharma & Anand, 1997). These compounds were seen having favorable activity against filariasis and schistosomiasis (Sharma & Anand, 1997). An infection with filariasis is caused by several species of parasitic roundworms present in blood, lymphatic tissue and other body tissues of the patient (Cobo, 2014; Nutman, 2017).

Modes of action

Nitrofurantoin destroys bacteria entering the body through multiple mechanisms such as DNA damage, RNA damage, protein damage as well as hindrance of the citric acid cycle (Blass, 2015). Nitrofurantoin can also kill anaerobic pathogens, because it is a hypoxic agent (Zuma *et al.*, 2020).

Nitrofurantoin resistance

Although nitrofurantoin has been used in clinical application for years, bacterial resistance have not been reported against nitrofurantoin, which makes nitrofurantoin logically the best option to manage gastrointestinal roundworms in livestock (Blass, 2015; Zuma *et al.*, 2019, 2020). Nitrofurantoin has multiple mechanisms to kill pathogens, this explains why there is a lack of bacteria forming resistance (Blass, 2015). For bacteria to develop resistance it undergoes natural selection and mutation to overcome one mechanism of action in the case of nitrofurantoin, bacteria has to overcome four mechanisms of action (Blass, 2015).

This drug represents a multi-activity drug, used in both agriculture and basic antibiotics therapies in human studies (Zuma *et al.*, 2019). Because of this multi-usage of the drug, it may present itself to be useful in the management of nematicidal activity in sheep and goats.

1.11 Aim

The study aims at assessing the effect of novel N-alkyl analogues of nitrofurantoin on the larval development and larval mortality of *C. elegans.*

1.12 Objectives

- To determine *in vitro* activity of different concentrations of N-alkyl analogues of nitrofurantoin on *C. elegans* nematodes.
- To determine *in vitro* activity of different concentrations of N-alkyl analogues of nitrofurantoin on the percentage inhibition of *C. elegans* larval development.
- To determine *in vitro* activity of different concentrations of N-alkyl analogues of nitrofurantoin derivatives on *C. elegans* survival rate.

1.13 Hypothesis

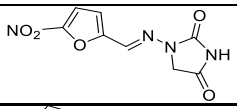
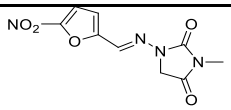
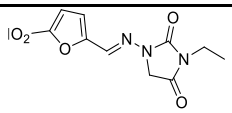
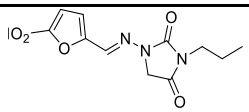
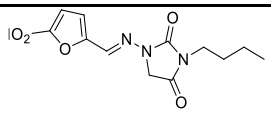
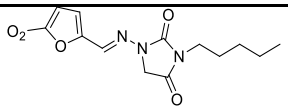
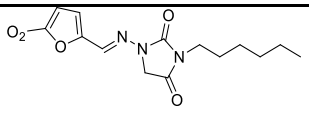
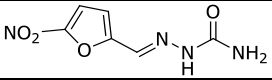
N-alkyl analogues of nitrofurantoin have nematocidal activity.

CHAPTER 2 MATERIALS AND METHODS

2.1 Compounds used in this study.

The nitrofurantoin derivatives, N-alkyl/aryl analogues (101-106) investigated in this study were previously synthesized by Zuma *et. al*. (2020) and are shown in Table 2-1.

Table 2-1: Nitrofurantoin N-alkyl analogues screened for nematocidal activity.

Compound	Structure	Molecular weight MW(g/mol)	Mass (mg)
Nitrofurantoin, **NFT**		238.16	10.7
101		252.18	9.6
102		266.21	9.9
103		280.24	10.1
104		294.26	10.6
105		308.29	10.4
106		322.32	10.4
Nitrofurazone, **NFZ**		198.14	9.9

2.2 Dissolving compounds with dimethyl sulfoxide (DMSO)

It was determined that the DSMO had to be mixed with phosphate buffered saline (PBS). Each compound was mixed with 10% DMSO in PBS. After a four-day incubation with just the buffer it was detected that the 10% DMSO in PBS mixture did not kill any *C. elegans* larvae and was suitable to dissolve the test compounds.

The nitrofurantoin analogues and clinically nitrofuran drugs (NFT & NFZ) in the table above were in a powder form and were dissolved in 10% DMSO/PBS mixture as follows:

The eight different nitrofuran derivatives mentioned in Table 2.1, were dissolved in a 10% DSMO/PBS solution. 10 mg of each compound was weighed out and transferred in a 2 ml Eppendorf tube. 100 µl of DSMO was added to the powdered compound for the compound to dissolve. The mixture was then vortexed. After the mixture was vortexed 900 µl PBS was added. This was done to all eight different nitrofurantoin analogues. Three different serial dilutions were then made from the original nitrofurantoin concentration. 100 µl of the original concentration was taken and transferred into a 2 ml Eppendorf tube. Thereafter 900 µl of PBS was added to the 100 µl of nitrofurantoin, lowering the concentration with each dilution as illustrated by Figure 4.

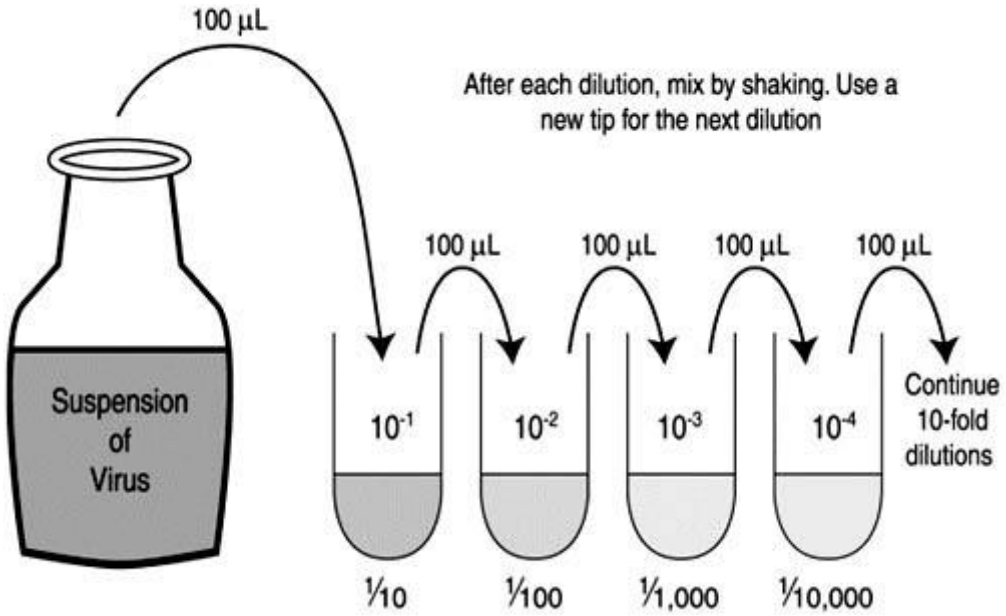


Figure 4: An example of how serial dilutions are made. The same method was used to dilute each compound and is represented by 10x-1, 10x-2, and 10x-3 in the results.

2.3 LB-medium and E. coli stock solution culture

LB- medium was made for a food source for *E. coli*. LB-medium was made by weighing out 0,5 g casein peptone, 0,25 g yeast extract and 0,5 g sodium chloride (NaCl) in a 250 ml flask. It was then dissolved with 50 ml double distilled water. A cotton ball (covered with foil) was placed at the opening of the flask and was autoclaved for 20 min at 121°C. When the LB-medium and nematode growth-medium agar was finished with the autoclave, a period of time was waited for both the mediums to cool down. When the LB-medium was cooled down enough to touch 20 µl of *E. coli* was added

E. coli was inoculated under sterile conditions 50 ml of LB-medium in a 250 ml Erlenmeyer flask with *E. coli* from a bacterial lawn on agar using an inoculating loop and incubated for 17 h at 37℃ on a shaker. Thereafter 200 µl glycerol was transferred into 1, 5 ml plastic vials and sterilize in an autoclave at 121℃ for 20 min. Under sterile conditions 800 µl of the *E. coli* overnight culture was added to the glycerol vials, vortex and frozen immediately at -20℃. Bacterial stock cultures are thawed only one and discarded after use. Bacterial stock cultures should be replenished after six months (ISO, 2008).

Nematode growth-medium agar (NGM agar)

To make the nematode growth-medium agar for the E coli mentioned in (2.3.3), 2,5 g of casein peptone, 17 g of bacterial agar, and 3 g of sodium chloride (NaCl) was weighted in a 1 000 ml DURAN reagents bottle. Double distilled water (900 ml) was added to dissolve the agar mixture, and was placed in the same autoclave as the LB-medium, for 20 min at 121°C.

When the LB-medium and nematode growth-medium agar was finished with the autoclave, a period of time was waited for both the mediums to cool down. After cooling down to 55°C the following sterile solutions was added to the nematode growth-medium agar; 1 ml of cholesterol stock solution, 1 ml of calcium chloride stock solution, 1 ml of magnesium sulfate stock solution, and 25 ml of potassium phosphate buffer. Cholesterol stock solution was made by adding 500 mg of powered cholesterol to 1 000 ml of absolute ethanol. The solution was dissolved by stirring it gently while heating it (no greater temperature than 50°C should be reached). Calcium chloride stock solution was made by adding 147 g of $CaCl_2 \cdot 2H_2O$ to 1 000 ml distilled water which was autoclaved for 20 min at 121°C. Magnesium sulfate stock solution (1 mol/$MgSO_4$) was made by adding 247 g of $MgSO_4 \cdot 7H_2O$ in 1 000 ml of distilled water which was autoclaved for 20 min at 121 °C. Potassium hydroxide buffer (1mol/KH 2 PO 4) was made by adding 136 g of KH_2PO_4 in 1 000 ml of distilled water and adjusted with potassium hydroxide (KOH pellets to achieve a pH of 6,0± 0,2, which was also autoclaved for 20 min at 21 °C. The KOH pellets were dissolved in the distilled water.

When all the stock solutions were added to the nematode growth-medium the DURAN reagents bottle was rolled to ensure that all of stock solutions mixed well with the agar. After mixing, 20 ml to 25 ml of agar was transferred to sterile Petri dishes. The agar plates were incubated upside down at a temperature of 37°C overnight.

2.4 Caenorhabditis elegans stock culture

Caenorhabditis elegans is a small, free-living nematode that can be found in soil that belongs to the phylum Nematoda (Byerly *et al.*, 1976; Palikaras & Tavernarakis, 2013). The adult worm can grow up to 1 mm with a diameter of 80 µm (Palikaras & Tavernarakis, 2013).

The life cycle of *C. elegans* is like that of common nematodes, compromising of the embryonic stage as well as four larval development stages and finally reproductive adults (Altun & Hall, 2006). This nematode contains an alternate dispersal life stage when its food source is exhausted, referred to as dauer (White *et al.*, 2019).

Caenorhabditis elegans was conserved and maintained on agar plates (NGM agar) with *E. coli* on the agar plates as a food source. When all the bacteria were consumed the *C. elegans* formed dauer larvae (stress stage) due to lack of food. These starved plates served as stock cultures for *C. elegans* that can be replaced every other month (International Organization for Standardization [ISO], 2008).

2.5 Pre-culture

Agar plates were inoculated under sterile conditions with approximately 200 µl of an overnight culture of E. coli, which was distributed equally using a Drigalski spatula and incubated for at least 8 h at 37°C.. Three small pieces (roughly 1cm^2) were cut out of a starved plate that contained *C. elegans* dauer larvae and were transferred under sterile conditions on to the agar plates with a fresh lawn of *E. coli.* After 3 days at 20℃, lot of gravid hermaphrodites as well as juveniles of stages 1 and 2 were found on the plates. The test started with worms in the first juvenile stage. To obtain worms synchronized to this life stage, the plates were rinsed with M9-medium, and the mixture was poured into a 50 ml centrifuge tube. The mixture was centrifuged at 4200 rcf for 3 minutes, thereafter the supernatant was discarded, leaving behind a pellet. The pellet was then exposed to the bleach out method. The bleach out method consisted out of 10 ml bleach that was added to a 50 ml centrifuge tube, thereafter, adding 40 ml of water from a Milli-Q ultrapure water system. Five pellets of sodium hydroxide were added to the bleach mixture. The pellet was left in the bleach mixture for six to seven minutes, (all the worms had to be dead) but not exceeding 11 minutes to minimize damaging the eggs. This mixture was centrifuged at 2400 rcf for 2 minutes and the supernatant was disgorged after. The pellet was then washed with M9-medium by adding the medium to the pellet and centrifuge the mixture at 2400 rcf for two to three minutes, this step was repeated twice. Finally, 15 ml M9-medium was added to the pellet and incubated at in a shaker at 120 rpm at 21℃ overnight. After incubation 30 first stage juveniles (J1) were obtained to observe.

2.6 Preparation of food medium

A vial with *E. coli* stock was vortexed and incubated under sterile conditions χ x 50 ml of LB-medium in a 250 ml Erlenmeyer flask with 20 µl of the *E. coli* stock each incubated for 17 h at 37℃ on a shaker. To control the bacterial density after incubation, the bacterial suspension was diluted 1 to 10 with LB-medium and then the optical density was measured at 600nm against the LB-medium. The turbidity of the bacterial suspension was given as specified in ISO 7027 (an ISO standard for water quality that enables the determination of turbidity) in formazine absorption units (FAU). After centrifugation of the bacterial suspension (20 min, 2000 g), the supernatant was removed. M9-medium was added to the pellet for resuspension.

2.7 In vitro assay

The 50 ml centrifuge plastic vial that was incubated overnight on the shaker was retrieved using a micropipette and used to isolate L_1 stage larvae and placed these individuals in a 12 well plate. Ten individual L_1 stage larvae were counted and placed in each well. To control the bacterial density after incubation at 21°C, an aliquot of bacterial suspension was diluted 1 to 10 with LB-medium, and the optical density was measured at 600 nm against the LB-medium. The optical density at 600 nm against the LB-medium . The turbidity of the bacterial suspension was given as specified in ISO 10872 in FAU. After the centrifugation was done of the bacterial suspension for 20 min ats 2 000 rpm , the supernatant was removed and suspended with M9-medium. After repeated centrifugation The supernatant was centrifuged once more for 20 min at 2 000 rpm. Afterwards the supernatant was removed, and the pellet was resuspended in 50 ml M9-medium.

Contents added to each well with ten L_1-stage larvae:

- 500 µl of *E. coli,* M9-medium mixture
- 350 µl of M9-medium
- 50 µl of Nitrofurantoin analogues solution.

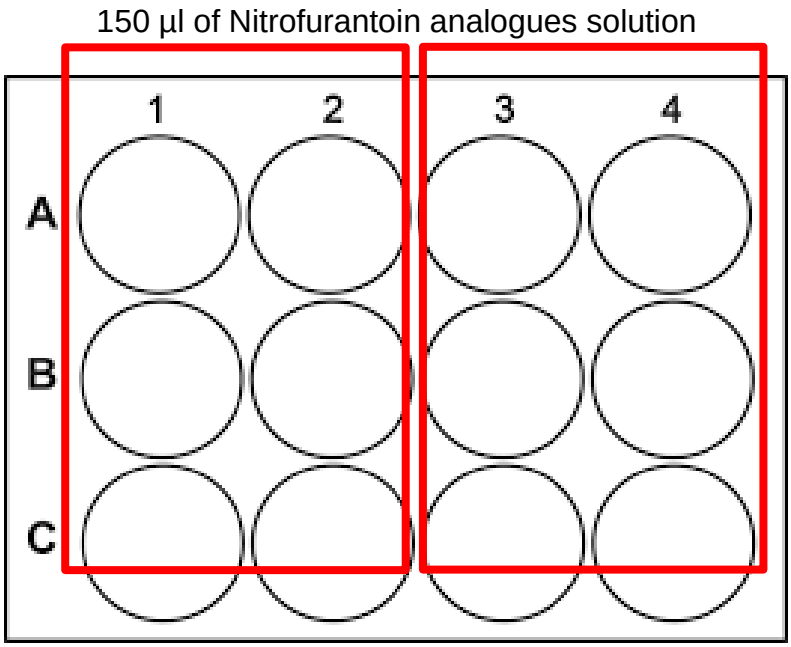


Figure 5: A diagram of a 12 well plate which was used for the *in vitro* assays. Each derivative was repeated five times.

Before larvae were added to the wells each of the eight different derivatives of the compounds were diluted three times by means of serial dilutions. Each dilution was then repeated five times. The derivatives were diluted as follows. After larvae was added to the wells (10 larvae in each well), the eight different compounds were added containing the larvae. All eight different derivatives were repeated 5 times in the 12 well plate. This made a 12 well plate suitable for the *in vitro* assay of two compounds as illustrated by Figure 5. The red boxes represent one compound. After all the compounds, test materials and food mediums were added the wells were placed in an incubator at 21°C for four days. When the incubation period was over approximately 0,5 ml of Rose Bengal stock solution was added to each test well to stain the nematode cuticle for better recovery and placed in a drying oven for 10 min at 80°C to terminate the test. Bengal rose stock solution was made by adding 300 g of Bengal Rose to 1 000 ml of distilled water which was thoroughly stirred. These samples were stored in a fridge at (8±2 °C) for 8 weeks. The nematodes were

transferred to a petri dish to observe under a stereo microscope. Double distilled water was used as the negative control, whereas ivermectin was used as the positive control. The *in vitro* assay described above was used for all three assays presented in the results.

The positive controls of the study were Ivermectin. Ivermectin was diluted the following way. An Eppendorf tube was filled with 900 µl distilled water and 100 µl Ivermectin. The negative control of the study was distilled water and was just used as is. Each control was repeated five times, just like the compounds.

CHAPTER 3 RESULTS

3.1 Background

The initial 10 larvae nematodes exposed in the *In vitro* assay had developed into adulthood in the four-day incubation period. These adults were counted, and an average of each replicate was calculated and the percentage of worms that died was found. The percentage of nematocidal activity was calculated by dividing the total number of recovered exposed test nematodes by the number of introduced test nematodes and multiplying by 100, illustrated by Table 3.1**.** Three different serial dilutions were made from the original nitrofurantoin concentration and are represented as 10x-1 (as the highest diluted concentration), and 10-3 as the lowest diluted concentration. This is the same for all three assays and are represented the same in all three tablesThe average of compound 104. 105, and 106 was calculated to determine the best performing nematocidal compound.

Average % of values from Table 3.1

Compound **104**: (98% + 94% + 72.544%)/3 = 88.182 % nematicidal effect.

Compound **105**: (100% + 96.362% + 91.362%)/3 = 95.908 % nematicidal effect.

Compound **106**: (100% + 92% + 96%)/3 = 96 % nematicidal effect.

Table 3-1: Nematocidal effect of compounds on nematodes

Compound	***C. elegans***			**Cytotoxicity**, IC_{50} (µM)
	10x-1	10x-2	10x-3	CHO cells
101	(0.96 mg/ml) 4.545 (%)	(0.096 mg/ml) 2 (%)	(0.0096 mg/ml) 50 (%)	>100
102	(0.99 mg/ml) 82 (%)	(0.099 mg/ml) 8 (%)	(0.0099 mg/ml) 23.45 (%)	60.4 µM
103	(1.01 mg/ml) 7.5 (%)	(0.101 mg/ml) 16 (%)	(0.0101 mg/ml) 72 (%)	40.2
104	(1.06 mg/ml) 98 (%)	(0.106 mg/ml) 94 (%)	(0.0106 mg/ml) 72.544 (%)	77.8 µM
105	(1.04 mg/ml) 100 (%)	(0.104 mg/ml) 96.362 (%)	(0.0104 mg/ml) 91.362 (%)	>100 µM
106	1.04 mg/ml 100 (%)	(0.104 mg/ml) 92 (%)	(0.0104 mg/ml) 96 (%)	>100 µM
NFT	(0.99 mg/ml) 96.362 (%)	(0.099 mg/ml) 98 (%)	(0.0099 mg/ml) 54 (%)	50.2 µM
NFZ	(0.99 mg/ml) 100 (%)	(0.099 mg/ml) 90.19 (%)	(0.0099 mg/ml) 88.23 (%)	97.2 µM
Ivermectin	(100%)	N.A.	N.A.	

Compound 106 (100%) had the highest percentage of nematocidal effect of the introduced L_1 larvae followed by NFZ (100%). Compounds 103 and 101 had the lowest percentage of nematocidal effect (7.5% and 4.54% respectively) on the introduced L_1 larvae. The highest concentrations of all the compounds had the highest percentage of nematocidal effect on the larvae.

Larval development inhibition is shown in Table 3-2 and it is indicated by the percentage of larvae that developed into adult worms during the incubation period of four days. Larval development inhibition was calculated by counting the number of worms that developed into adults during the incubation period (four days). The percentage of the larvae was then calculated by means of dividing the total number of adult's nematodes by the number of introduced test nematodes at the beginning of the four-day incubation period. This equation was then multiplied by 100 to achieve the % of larval development inhibition. Compounds 101 (98%) and 102 (94, 5%) had the highest percentage of larvae that developed into adults according to Table 3-2, whereas compound 106 (0-8%) had the lowest percentage of larval development.

The avearge of the best performing compounds were calculated to determine the most optimal compound for larval inhibition development.

Average % of values from Table 3.2

Compound **104**: (2 + 6 + 27.4)/3 = 11.76 % larval development.

Compound **105**: (0 + 1.8 + 7)/3 = 2.93% larval development.

Compound **106**: (0 + 8 + 4)/3 = 4 % Larval develpment.

Table 3-2: Percentage (%) inhibition of larval development

Compound	*Percentage inhibition of C. elegans larvae*		
	10x-1	10x-2	10x-3
101	92.3	98	42
102	25.6	92	94.5
103	94	84	28
104	2	6	27.4
105	0	1.8	7
106	0	8	4
NFT	3.6	2	47
NFZ	0	9.6	10
Ivermectin	0	0	0

When looking at the first concentration, Table 3-3 illustrates that concentration 10x-1 of compounds 101 and 103 had the highest amount of larvae (1010 and 2513 respectively) as a result of the adult worms that reproduced, whereas compound 104 (18 juveniles hatched but were dead) had the lowest amount of juveniles that hatched. Reproduction of adult nematode worms were calculated by counting the amount of juvenile (J1 stage) worms that were present with the initial 10 nematodes that developed into adults during the four day incubation period. Some juveniles survived while others did not, which is indicated with the words "survived" or "dead".

Table 3-3: Survival rate of adult *C. elegans*

Compound	*C. elegans*		
	10x-1	10x-2	10x-3
101	1010.0 (1010 survived)	991.0 (991 survived)	251.0 (251 survived)
102	155.0 (155 dead)	1633.0 (1633 dead)	1320.0 (1320 survived)
103	2513.0 (2513 survived)	2256.0 (2256 survived)	897.0 (897 survived)
104	18.0 (18 dead)	19.0 (19 dead)	24.0 (24 survived)
105	11.0 (11 dead)	27.0 (27 dead)	35.0 (35 survived)
106	18.0 (18 dead)	29.0 (29 survived)	28.0 (28 survived)
NFT	30.0 (30 dead)	23.0 (23 survived)	34.0 (34 survived)
NFZ	20.0 (20 dead)	31.0 (31 survived)	28.0 (28 survived)
Ivermectin	0	0	0

CHAPTER 4 DISCUSSION

The *C. elegans* is considered as a simple but important invertebrate model which provides a versatile platform of various of experiments such as drug studies, (Connor & Watts, 2019; Hahnel *et al.*, 2020; Muschiol *et al.*, 2009; Rabinowitch & Schafer, 2017; Ro & Rosenzweig, 2018) and contain multiple advantages for such studies because of their short life cycles, small sizes, large number of offspring, and their simplicity of cultivation (Artal-Sanz *et al.*, 2006; Carranza-García & Navarro, 2020; Corsi *et al.*, 2015). *C. elegans* is used in various of studies to represent the whole phylum Nematoda, because they share the main characteristics of the nematode body plan (Geary & Thompson, 2001; Gilleard, 2004; Hahnel *et al.*, 2020; Holden-Dye & Walker, 2014; Riddle *et al.*, 1997; Salinas & Risi, 2018).

Nitrofurantoin, hydration and metronidazole are part of the nitroheterocyclic compounds which are commonly used for infectious diseases in the medical field (Hof, 1988; Vardanyan & Hruby, 2006). Nitrofurantoin has shown to have a powerful antimicrobial effect, especially on various of bacterial groups, protozoa and worms (Hof, 1988; Sharma & Anand, 1997). This drug is used for various of treatments for human purposes, for example from urinary tract infections (pyelitis, pyelonephritis, cystitis, urethritis) to treating certain cancers (Howard, 2007; Thakuria *et al.*, 2017; Vardanyan & Hruby, 2006; Zuma *et al.*, 2019).

Even though nitrofurantoin is mainly used for its antibacterial activity, it is noted that certain compounds have been able to kill microfilariae and the adult worms of *Litomosoides carinii* (Sharma & Anand, 1997). These compounds were seen having favorable activity against Filariasis and schistosomiasis (Sharma & Anand, 1997). An infection with filariasis is caused by several species of parasitic

roundworms present in blood, lymphatic tissue, and other body tissues of the patient (Cobo, 2014; Nutman, 2017).

Nitrofurantoin destroys bacteria entering the body through multiple mechanisms such as DNA damage, RNA damage, protein damage as well as hindrance of the citric acid cycle (Blass, 2015). One of these mechanisms involves nitrofurantoin (NFT) as a hypoxic agent and has activities involving killing anaerobic pathogens (Zuma *et al.*, 2020). When placed in conditions with oxygen, nitro reduction catalyzed by a type 2 NTRs give rise to reactive oxygen as well as nitrogen species (Zuma *et al.*, 2019). This leads to oxidative stress to pathogens which leads to death (Zuma *et al.*, 2019).

It is known that nitrofuran drugs has been in clinical application for more than 60 years (Zuma *et al.*, 2019, 2020). Despite its use for years, bacterial resistance according to reports are rare which makes it logically the best option to use (Blass, 2015). The characteristic of nitrofurantoin that allows for multiple mechanisms to kill bacteria explains the lack of resistance forming (Blass, 2015). For bacteria to develop resistance it undergoes natural selection and mutation to overcome one mechanism of action in the case for example of penicillin, nevertheless, in the case of nitrofurantoin, a bacteria has to overcome four mechanisms (Blass, 2015).

This drug represents a multi-activity drug, used in both agriculture and basic antibiotics therapies in human studies (Zuma *et al.*, 2019). Because of this multi-usage of the drug, it may present itself to be useful in the management of nematocidal activity in sheep and goats.

Compound 101 in Table 3.1 did not have a nematocidal effect on the roundworms due to the large number of worms that survived after the incubation period of four days.This was indicated by the low percentage of 4.54% in Table 3.1. But concentration 0.0096 mg/ml was seen to have the most nematocidal effect on the worms which had a percentage of 50%. Larval development was not affected by compound 101 as shown in Table 3.2. This means that the initial 10 juvenile nematodes that were exposed to the compound and was left to incubate for four days 92.3% of the nematodes grew into adults with no harm done to them. The number of juvenile offspring is also high after the four-day incubation, as indicated by the number of juveniles (1010 juveniles) when considering the first concentration (0.96 mg/ml) in table 3.3. This indicates that the compound had no effect on the reproduction system of adult worms, as well as no effect on juvenile worms. This compound was not taken into further evaluation due to lack of nematocidal effect on the round worms.

Analogue 102 had a nematocidal effect on the roundworms during the four-day incubation period. The highest concentration (0.99 mg/ml) of compound 102 revealed to be the best option for nematocidal compound, due to the large number of worms that died/disintegrated (82%) during the four-day incubation period revealed by table 3.1. Compound 102, as illustrated by table 3.2, did not inhibit the development of the 10 initially introduced larvae, because 25% of the larvae developed into adults from the strongest concentration. It is also noted that the highest concentration of compound 102 produced the least number of offspring/juveniles (155) during the incubation period. Although there where juveniles present, when observations took place of the worms after the incubation period, all the juveniles where dead. Whereas in the lower concentration, the

juveniles present were more, and after incubation, the juveniles were observed to be alive.

When observing the results of compound 103 it is clear that this compound and compound 101 have more or less the same results. Analogue 103 did not have a nematocidal effect on the roundworms due to the large number of worms that lived after the incubation period of four days, indicated by table 3.1. But concentration 0.0101 mg/ml was seen to have the most nematocidal effect on the worms causing 72% of the initial population to die. Table 3.2 indicates that compound 103 had little effect on the inhibition of the larval development as 94% of the introduced population developed into adults. The number of juvenile offspring is also high after the incubation period, as indicated by the number of juveniles (2513 juveniles) that survived shown in table 3.3. This indicates that the compound had no effect on the reproduction system of adult worms, as well as no effect on juvenile worms. This compound will not take be taken into further evaluation due to lack of nematocidal effect on the round worms.

Compound 104 had significant nematocidal effects on the roundworms during the incubation period. When observing the tables above, it is clear that as the concentration of the compound lowered from 1.06 mg/ml to 0.0106 mg/ml, so did the number of dead nematodes. The highest concentration of the compound (1.06 mg/ml) had the highest number of dead nematodes (98% of the population died). Larval development was successfully inhibited with only 2% of the population surviving the incubation period, illustrated by table 3.2. During the experiment 49 out of 50 of the introduced nematodes died/disintegrated. Lowering the concentration to 0.106 mg/ml caused less nematodes to die and more offspring was

counted. The lowest concentration, 0.0106 mg/ml, had the least number of nematodes that died, and highest number of offspring (24) was counted. This compound can therefore be considered as a nematocidal compound.

Compound 105 indicated that this compound has nematocidal tendencies, because of the death/disintegration of all 50 worms introduced to the highest concentration (1.04 mg/ml) during the four-day incubation period. As the concentrations become lower, from 1.04 mg/ml to 0.104 mg/ml to 0.0104 mg/ml, more nematodes were found to be alive after the incubation period. Larval development was also successful as 0% of the population grew into adult nematodes, illustrated by table 3.2. The number of offspring/juveniles also increase as the concentration decreases. Because of the results of compound 105, it can be considered as a nematocidal for round worms.

Compound 106 at the highest concentration kills 100% of the introduced worms during the incubation period. Compound 106 also inhibited larval development as indicated by table 10.2, as 0% of the introduced larvae of the highest concentration did not develop into adults. At the concentration of 1.04 mg/ml the table also indicated that 18 offspring/juveniles were observed, but they were upon exception dead. As the concentration decreases to 0.0104 mg/ml, the number of offspring rises as well as the number of nematodes that are still alive after four days of incubation. Because of the results, compound 106ncan be considered as a nematocidal for nematodes.

When considering compound NFT it is clear that this compound had a nematocidal effect on the roundworms during the four-day incubation period. The highest concentration (0.99 mg/ml) and the second concentration (0.099 mg/ml) of

compound NFT revealed to be the best option in a nematocidal, due to the large number of worms that died/disintegrated during the four-day incubation period, 49 worms dead out of 51 introduced worms. It is also noted that the concentration 0.099 mg/ml produced the least number of offspring/juveniles during the incubation period. The juveniles that were observed after the four-day incubation, were all alive. Therefore, the compound had no nematocidal effect on the juveniles. That is why NFT was not considered as an option for a nematocidal for gastrointestinal nematodes.

NFZ had significant nematocidal effects on the roundworms during the four-day incubation period. The highest concentration of the compound (0.99 mg/ml) had the highest number of dead nematodes. During the experiment 51 out of 51 of the introduced nematodes died/disintegrated. Lowering the concentration to 0.099 mg/ml caused less nematodes to die and more offspring was counted. The lowest concentration, 0.0099 mg/ml, had the least number of nematodes that died, and highest number of offspring (28 juveniles) was counted. This compound can therefore be considered as a nematocidal compound.

The nitrofurantoin analogues 102, 104, 105, and 106 proved to have a nematicidal effect on *C. elegans* larvae. The average of table 3.1 was calculated for compound 104, 105, and 106 to establish which compound performed the best. The average was calculated by adding each percentage of the three different concentration, then dividing by the count of those percentages.

By considering the averages calculated above in Table 3.1, it is clear that compound 106 had the strongest nematicidal effect, killing 100% of the population at 1.04

mg/ml, 92% of the population at 0.104 mg/ml, and finally 96% of the population at 0.0104 mg/ml, with a 96% nematicidal effect overall. The positive control, Ivermectin, had a 100% nematocidal effect on the nematodes. None of the initial 10 introduced L1 stage larvae survived the four day incubation period.

It is clear that compounds 104, 105, and 106 had inhibited larval development the best. The average of table 3.2 was calculated for compound 104, 105, and 106 to establish which compound performed the best. The average was calculated by adding each percentage of the three different concentration, then dividing by the count of those percentages.

The worst performing compounds that did not inhibit the larval development during the essay was compound 101 and 103. After the incubation period of four days it was noted with compound 101, and 103 that the 10 innitial L1 stage larvae that was introduced at the beginning of the test survived and had the ability to reproduce sucsessfully. Compound 105 was found to inhibit the development of the larvae the most effective from the overall averages calculated above. Only 2.93% of the introduced nematodes fully developed into adulthood. When considering NFT and NFZ, it is clear that NFZ was the best performing compoundcompared to NFT. The positive control, which was Ivermectin, had the same percentage larval development inhibition, indicating that the controls that were choosen was good.

When considering table 3.3, the compound that produced the least amount of offspring was calculated by just simply adding the amount of offspring for each concentration, and seeing which compound produced the least number. It was found that compound 104 produced the least number off offspring, not only this,

compound 104 had the highest number of dead offspring between compounds 105 and 106, which is why compound 104 was the most effective compound when considering reproduction of *C. elegans*.

Compound 101, indicated that a large number of larvae nematodes did not die, even with the highest concentration, which was 1010 juviniles that survived the assay, in a four day incubation period. Compound 102 had fascinating results, because the 10 introduced L1 stage larvae did grow and reproduce, but it was interessting to note that all the offspring had died in the four day incubation period, whereas in compound 101 the offspring survived. Compound 103 had similar results as 101, the amount of offspring produced by the initial 10 L1 stage larvae was just observed to be more that compound 101. When comparing NFT and NFZ, it is observed that NFT was the best performing compound when looking at the survival rate of *C. elegans*. NFT initial 10 L1 stage larvae produced less offspring than NFZ. The positive control, Ivermectin, had the best results, as none of the initial 10 L1 stage larvae developed and there was no offspring present in the wells.

However, the cytotoxicity data in Table in 3-1, revealed that nitrofurantoin analogues 101, 105 and 106 indicate a low cytotoxicity (IC_{50} >100 µM) against CHO cells as indicated by (Fu *et al.*, 2014). Hence, the antiparasitic action of these three compounds is deemed intrinsic, indicating that the observed biological effects are not due to general toxicity of the compounds but rather their structures. Analogues 102, 104 and NFZ, on the other hand, with 50< IC_{50} <100 µM, are weakly toxic hence their antiparasitic action are partially due to their general toxicity as indicated by (Liu *et al.*, 2017). Lastly, nitrofuran derivative 103 and the clinical antibiotic, NFT

are deemed mild-to-moderately toxic (10 < IC_{50} < 50 µM) (Liu *et al.*, 2017). As such, with its partial toxicity, analogue 104 can't be considered as the best performing compound of the study based on its safety profile. The choice for such an attribute should be among the safest analogues, i.e., 102 and 104, and NFZ.

compound 104 toxicity level is too high when looking at Table 3.1, this means thatthe next best performing compound needs to be choosen when it comes to killing the offspring of the adults nematodes. Compound 105 indicates in Table 3.3 that this compound killed more adult offspring than compound 106. Compound 105 also had more dead juveniles than alive ones.

During this study, it was discovered that the compounds were difficult to dissolve in dimethyl sulfoxide (DMSO), which is considered as a preferred solvent that can be used to dissolve not only polar compounds, but non-polar compounds as well (Choulis, 2012). The compounds were mixed with 1 ml pure DMSO and vortexed for 30 seconds. After the solution was vortexed it was noticed that the compound did not dissolve correctly, and clumps of the compound could be observed. Not only did the compound not mix correctly, but after a four-day incubation period it was discovered that the DMSO concentrations were too harsh for the delicate *C. elegans* as all of the test worms were dead. It was determined that the DSMO had to be mixed with another buffer to solve this issue. This buffer was phosphate buffer saline (PBS). Each compound was mixed with 10% DMSO/PBS, this means that 900 µl was PBS, whereas 100 µl was DMSO. After a four-day incubation with just the buffer it was detected that the 10% DMSO in PBS mixture did not kill any *C. elegans* larvae and was suitable to dissolve the compound.

CHAPTER 5 CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

The objectives of the study were to determine i*n vitro* activity of different concentrations of N-alkyl analogues of nitrofurantoin derivatives on *C. elegans* larval development as well as larval mortality assay. Eight different N-alkyl derivatives of nitrofuran analouges were used, and can be identified as compound nitrofurantoin, 101 - 106, and nitrofurazone.

The nitrofurantoin analogues 104, 105, and 106, had the best nematocidal effect on the round worms, with 106 being the most effective at killing the roundworms. However, 105 demonstrated the best larval development inhibition whereas 104 produced the least number of L_1 stage larvae and the highest number of dead larvae, but due to the toxicity levels of compound 104, compound 105 can be seen as the best option when it comes to killing offspring of adult nematode worms. This means that compound 105 caused *C. elegans* to reproduce less offspring than the other compounds.

The results are quite fitting, as each compound comes from the same parent drug, each compound has its unique qualities and characteristics that separate them from one another. This means that when one searches for a compound that has the best nematocidal effect, one would choose compound 106. But when one needs a compound to hinder the larvae's development, one would choose compound 105. And finally, when one needs a compound to kill the offspring of the adult worms, one would also choose compound 105. It all depends on what the need is at that point. What is important is that both compounds 105 and 106, disturbed or disrupted

the worms life cycle at a certain stage, which is considered a good management quality (Simpson, 2015).

The findings of this study are important and significant because parasitic gastrointestinal roundworms have a devastating impact on livestock worldwide. These parasites have the ability to develop resistance to anthelmintics that are currently in use. This means that the discovery of any compound that can possibly be used in the successful management of parasitic gastrointestinal roundworms are imperative.

5.2 Recommendations

During this study, it was discovered that the compounds did not dissolve easily in DMSO. Future studies should evaluate other possible solvents for these nitrofurantoin compounds.

The process of cultivating *C. elegans* was done according to [ISO] International Organization for Standardization, 2008-Water quality—Determination of the toxic effect of sediment and soil samples on growth, fertility, and reproduction of *Caenorhabditis elegans* (Nematoda). The shortcoming of this method is that it is best used to test the determination of the toxic effect of sediment and soil samples on growth, fertility, and reproduction of *C. elegans*. has a few drawbacks when it comes to determination of nematocidal effects of pharmaceutical compounds on *C. elegans*. This was especially noticed after the four-day incubation period, when the larvae were counted.

It was realized that instead of five repeats of one compound, the concentrations could have been increased in order to fully see the effect of the compounds, and with that an LD50 (lethal dose) graph could have been plotted to observe the concentration at which 50% of the test population is killed. In this study, only three different concentrations were used, which were not enough to plot an LD50 graph.

It is further recommended that toxicity studies of these compounds should be conducted on experimental animals.

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