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Addressing anthelmintic resistance in *Haemonchus contortus* strains in small ruminants through alternative strategies with a focus on *Moringa oleifera* extracts

by

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Declaration

I, Johannes A Dreyer hereby declare that this dissertation, submitted for the MSc (Agric) Animal Science: Animal Nutrition degree at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at any other University.

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Abstract

Anthelmintic resistance poses a significant threat to small-stock farming worldwide, with *Haemonchus contortus* being a major contributor to production losses and the deterioration of animal health. This study aimed to evaluate the potential use of *Moringa oleifera* (*M. oleifera*) in livestock production systems to combat Haemonchosis and anthelmintic resistance in small stock farms. This growing crisis threatens the sustainability of small-stock production, particularly in tropical regions where parasitic loads are high. One promising alternative to combat Haemonchosis is the use of phytochemical compounds derived from *M. oleifera*. *In vitro* inhibitory effects of *M. oleifera* on *Haemonchus contortus* larval motility and egg hatchability have been previously reported in literature.

In the current study ethanolic extracts from commercially sourced *M. oleifera* leaf and seed samples were analysed for phytochemical composition through UPLC-UV/QTOF/MS. Previous research reported that terpenoids, cinnamic acids, alkaloids, flavones and saponins are effective biochemical compounds against *Haemonchus contortus*. The analysis of the crude *M. oleifera* leaf and seed extracts revealed the presence of potentially bioactive menthane monoterpenoids, secoiridoid monoterpenoids and cinnamic acids like cimicifugic acid. This highlighted the potential for *M. oleifera* to be included in management strategies against Haemonchosis in livestock.

The *M. oleifera* extracts were further fractionated into seven (leaves) and five fractions (seeds) according to polarity and evaluated for their effects on larval motility and egg hatchability *in vitro*. For *M. oleifera* leaves, the average crude extract egg mortalities were recorded as 89.0%, 76.7%, and 100.0% for concentrations of 5, 10, and 15 mg/ml respectively. Similarly, the average crude extract larvae mortalities were 30.1%, 51.8%, and 54.3% for the same concentrations. Regarding *M. oleifera* seed extracts, the average egg mortalities were 80.7%, 95%, and 76.5% for concentrations of 5, 10, and 15 mg/ml respectively. Correspondingly, the average crude extract larval mortalities were 25.6%, 25.1%, and 28.9% for the same concentrations. Fraction 6 from *M. oleifera* leaves and fractions 5 and 3 from seeds exhibited the highest mortality percentages for both egg hatch and larval survivability assays, reaching up to 96.9% and 94.5% for leaves, and 94.6% and 71.4% for seeds respectively.

By exploring innovative alternative approaches to address the challenge of anthelmintic resistance, this study aimed to potentially enhance the resilience and sustainability of the sheep production sector, concurrently fostering animal health and welfare.

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List of Abbreviations

CE	Crude extract
CH_4	Methane
CSG	Concentration solution group
DM	Dry matter
DMOSM	Defatted *M. oleifera* Seed Meal
DMSO	Dimethylsulphate
DW	Distilled water
EBV	Epstein Barr virus
EHA	Egg hatch assay
F1	Fraction 1
F2	Fraction 2
F3	Fraction 3
F4	Fraction 4
F5	Fraction 5
F6	Fraction 6
F7	Fraction 7
FAMACHA	Faffa Malan chart
FECRT	Faecal egg count reduction test
GIN	Gastrointestinal nematodes
HIV	Human immunodeficiency virus
HSV-1	Herpes simplex virus type 1
L1	Free living larvae stage 1
L2	Free living larvae stage 2
L3	Free living larvae stage 3
L4	Free living larvae stage 4
LMA	Larval motility assay
LSA	Larval survivability assay
MeOH	Methanol
Mo:EtOH	*M. oleifera* ethanol extract
PGE	Parasitic gastroenteritis

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PTFE	Polytetrafluoroethylene
PUFA	Polyunsaturated fatty acid
SBM	Soybean meal
SFA	Saturated fatty acids
SPE	Solid phase extraction
UPLC-UV/QTOF/MS	Ultra-high pressure liquid chromatography – ultra-violet/quadrupole time-of-flight/mass spectrometry
WAAVP	World Association for the Advancement of Veterinary Parasitology

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Chapter 1: Introduction

1.1 Introduction

Gastrointestinal nematodes (GIN) continue to be one of the most significant challenges for ruminant production systems (Cabardo Jr and Portugaliza, 2017) in many tropical developing countries (Rupa and Portugaliza, 2016). Among the multiple of GIN species, *Haemonchus contortus* stands out as a major contributor to economic losses and animal welfare concerns due to its pathogenicity in small ruminants (Mortensen *et al.*, 2003) and ability to develop resistance to anthelmintic treatments (Cabardo Jr and Portugaliza, 2017).

Haemonchus contortus (*H. contortus*), commonly known as the barber's pole worm, is notorious for its blood-feeding behaviour and its ability to cause severe anaemia, leading to reduced productivity and even mortality in small ruminants (Bowman, 2009). The escalating problem of anthelmintic resistance, amplified by years of indiscriminate drug use (Kaplan, 2013) has become a pressing issue in the sheep production industry in tropical and temperate areas worldwide (Emery *et al.*, 2016). South African sheep production systems are regarded as one of the important livestock sectors delivering two main products nationally, namely meat (lamb and mutton) and wool (DAFF, 2020), with the national sheep flock of South Africa estimated to be approximately 22 million with an approximate 177 000 tons of meat produced per annum (DAFF, 2020). The husbandry of sheep is an important aspect to small stock production systems and is a critical source of income to the farmer as well as a source of meat and fibre to the industry (Getahun, 2008; FAO, 2009; Yisehak *et al.*, 2014; Alemu *et al.*, 2014). *Haemonchus contortus* is seen as one of the most significant hindrances to sheep production in South Africa (Vatta *et al.* 2001), thus the impact of GIN infections and resistance thereof on production efficiency cannot be overstated.

Historically, control measures against GIN relied heavily on synthetic anthelmintics and the overuse and misuse of these drugs have led to widespread resistance (Adamu *et al.*, 2013), especially in *H. contortus*. This resistance phenomenon has resulted in dwindling treatment options, escalating costs (Soulsby, 1986; Kassai, 1999), and compromised animal welfare (Allonby and Urquhart, 1975). Certain pharmaceutical companies have reported costs of antiparasitics to be approximately in the tens of billions of dollars worldwide (Wolstenholme *et al.*, 2004). Consequently, there is an urgent need to explore alternative strategies that can effectively manage GIN infections while mitigating the development of resistance.

Plant alternatives have emerged as promising candidates for combating anthelmintic resistance (Vargas-Magaña *et al.* 2014). Certain phytochemicals present in various plant species possess

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anthelmintic properties, offering a sustainable and environmentally friendly approach to parasite control (Ali *et al.*, 2021). Among these botanical options, *M. oleifera*, commonly known as the drumstick tree or horseradish tree, has amassed significant attention for its diverse medicinal properties (Gupta *et al.*, 2018).

Moringa oleifera (*M. oleifera*) has been described as a small, evergreen, and fast-growing deciduous tree (Mishra *et al.*, 2011). *Moringa oleifera* is native to the western and southern parts of the Himalayas, Afghanistan, and Pakistan and is widely cultivated in certain parts of Africa including Nigeria, Senegal, and Tanzania (Bosch, 2004; Salem and Makkar, 2009; Radovich, 2009). *Moringa oleifera* is now a native plant in most African countries, as well as in the Caribbean Islands and in Central America (FAO, 2014). A *M. oleifera* tree is considered as one of the world's most useful plants, as almost every part of the plant can be used to manufacture food, medicine, and industrial products (Khalafalla *et al.*, 2010). *Moringa oleifera* possesses different essential nutrients with a variety of phytochemicals present in its leaves, seed capsules and seeds (Gopalakrishnan *et al.*, 2016). Recent research discussed below has unveiled its potential as an anthelmintic agent, highlighting its efficacy against various gastrointestinal parasites, including *H. contortus*.

The anthelmintic properties of *M. oleifera* are attributed to its rich phytochemical composition, which includes bioactive compounds such as flavonoids, alkaloids, tannins, terpenes and saponins (Tayo *et al.*, 2014; Gopalakrishnan *et al.*, 2016; Romero-Benavides *et al.*, 2017; Cabardo Jr and Portugaliza, 2017; Ali *et al.*, 2021). These constituents exhibit diverse biological activities, including anthelmintic, antioxidant, and anti-inflammatory effects, making *M. oleifera* a promising candidate for sustainable parasite control in small ruminants.

1.2 Aim

In this study, the aim was to explore the potential of *M. oleifera* as an alternative anthelmintic agent against *H. contortus* and to investigate the underlying mechanisms of its anthelmintic activity. Through a comprehensive evaluation of the phytochemical profile and *in vitro* anthelmintic activity of the species, the study aimed to progress research towards exploring the feasibility of integrating *M. oleifera* into sustainable parasite management strategies for small ruminant production systems. By addressing the pressing issue of anthelmintic resistance through innovative alternative or complementary solutions, this research will venture to potentially contribute to the resilience and sustainability of the sheep production industry while promoting animal health and welfare. This study hypothesizes that specific bioactive compounds present in *M. oleifera* extracts will exhibit significant *in vitro* anthelminthic activity by

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disrupting specific metabolic pathways of *H. contortus*, thereby offering a viable, natural alternative to conventional anthelmintics in small ruminant parasite control.

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Chapter 2: Literature Review

2.1 A review of *Moringa oleifera*

Moringa oleifera Lam. belongs to the family Moringaceae. There are about 33 species in the Moringaceae family with the best-known species among this family including *M. arborea*, *M. borziana*, *M. concanensis*, *M. drouhardi*, *M. hildebrandtii*, *M. longituba*, *M. oleifera*, *M. ovalifolia*, *M. peregrina*, *M. pygmaea*, *M. rivae*, *M. ruspoliana and M. stenopetala*, which are found world-wide (Arora *et al.*, 2013).

Moringa oleifera is known by many names for instance; Miracle-, Horseradish- or Drumstick tree and is used in most of the tropical regions worldwide (Jahn, 1988). It is a small to medium sized tree, fast growing and has a wide range of uses, ranging from forage for livestock, nutrition, medicinal uses, purification of water, and cosmetic products (Fuglie, 2001, Anwar *et al.*, 2007, Rashid *et al.*, 2008, Muhl, 2014). This tree can grow from sea level to upwards of about 600 m above sea level and is also found in the hills of the Himalayas (1000 m above sea level), the plains of West Africa (1350 m above sea level) and even as high as 2000 m above sea level in Zimbabwe (Bosch, 2004; Radovich, 2013).

Moringa oleifera grows well in areas where the average temperature is between 25º and 30ºC (Radovich, 2013). Low temperatures together with frost can cause damage down till the ground level of the plant but it will regrow fast and efficiently once the temperature increases, and it grows well in full sunlit areas (Foidl *et al.*, 2001). These trees thrive in areas with an annual rainfall between 1000 and 2000 mm but are also very tolerant of drought, growing in areas with annual rainfall as low as 400 mm (Muhl, 2014).

The *M. oleifera* tree grows to heights of 10-12 m, with an umbrella-shaped, open spread crown. The roots grow deep with the base of the tree being crooked and one-stemmed. The bark resembles the appearance of a cork and is grey in colour (Radovich, 2013) as shown in Figure 2.1. *M. oleifera* leaves grow in an alternate arrangement of 7-60 cm in length with a green to dark green colour (Orwa *et al.*, 2009).

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Figure 2. 1: *Moringa oleifera* tree, seeds and leaves.

The zygomorphic flowers have a cream to white appearance and are pollinated by a vast number of different insects (Bhattacharya and Mandal, 2004). The fruit of *M. oleifera* comprise three lobed pods, 20-60 cm, which hang from the tree branches. When the fruits become dry, they separate into three compartments with each pod containing 12-35 seeds each (Bhattacharya and Mandal, 2004). The seeds are globular shaped with a brown hue and a semi-permeable seed hull (Figure 2.1).

Each *M. oleifera* tree has the potential to yield 15 000 to 25 000 seeds per year (Foidl *et al.*, 2001), with an average seed weighing 0.3 g (Makkar and Becker, 1997). *Moringa oleifera* can tolerate a broad range of soil conditions, but desires more neutral to marginally acidic soil with a pH of 6.3-7.0, with well drained loamy or sandy soil (Thurber and Fahey, 2010).

Moringa oleifera has a harvesting rate of approximately 2.5 months with proposed cutting intervals of between 15 and 75 days. This yields between 27-120 tons/hectare (t/ha) of fresh dry matter at first cutting (Amaglo *et al.*, 2007). However, this can lead to variable results, as a study conducted in Taiwan showed a high dry matter yield of 4.2-8.3 t/ha where different levels of fertilizer were used (Palada *et al.*, 2007). Foidl *et al.* (2001) deduced that with 1 million plants planted per hectare and 9 cuttings per year over a 4-year period, *M. oleifera* can produce approximately 99 t/ha of dry matter under intensive farming conditions. In terms of growth rate, *M. oleifera* produces the highest yields when it is subjected to warm and dry conditions with supplementary irrigation in drier conditions and approximately 300 grams of complete fertilizer (Radovich, 2013), which enables up to 9 cuttings per year (Foidl *et al.*, 2001).

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Moringa oleifera trees can be cultivated from pod production activities and can be harvested after 7 months prior to planting. These harvests can have yields of approximately 30 tons of pods per ha per year (Radovich, 2009). Bosch (2004) reported that seed production studies done in Tanzania reported a yield of approximately 3.3 kg of seeds per tree/year, which corresponds to about 1-1.5 t/ha of seed oil. Studies have shown that *M. oleifera* can be grown and produced in an environmentally sustainable way and with decreased cost to produce fodder.

In Figure 2.2, the utilization of *M. oleifera* is illustrated. It can clearly show the components of the plant that can be used as well as variety of applications ranging from consumption by livestock and humans as well as for cooking, medical and industrial products (Foidl *et al.*, 2001).

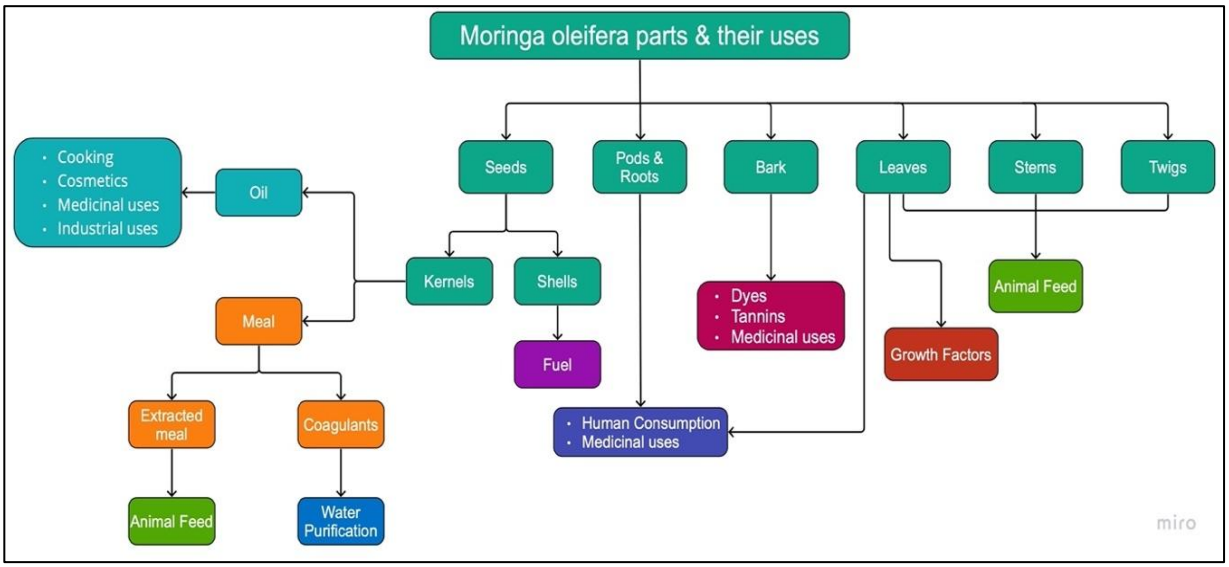

Figure 2. 2: *Moringa oleifera* and its uses (Adapted from Foidl *et al.*, 2001)

2.1.1 Medicinal value of *Moringa oleifera*

Moringa oleifera is praised for its economical and medicinal merits worldwide. It received the "Botanical of the Year" prize in 2007 by the National Institute of Health (NIH). It has many common names, but most prominent in Africa are "Never Die" or "Miracle tree" due to its ability to cure and help with over 300 diseases (Gupta *et al.*, 2018). *Moringa oleifera* has long been used as an herbal medicinal product in both India and Africa due to the many phytochemicals present in the plant (Gopalakrishnan *et al.*, 2016). Almost every part of this tree including the leaves, stems, root, bark, seeds, and flowers as well as seed oil and leaf extracts are believed to yield active ingredients useful in indigenous medicine (Fozia *et al.*, 2012). The various bioactive components researched in different parts of the *M. oleifera* tree represent a promising development in modern medicine and are portrayed in Table 2.1 below.

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Table 2. 1: The multiple medicinal properties of *Moringa oleifera* and the phytochemical compounds associated with the activities

Medicinal properties	Plant parts	Associated phytochemicals	Reference
Antioxidant activity	Leaves Seeds Bark Flowers Seed oil	Isothiocyanates, polyphenols and rutin Tocopherols, myricetin and lectins Procyanidins Palmitic acid, phytosterols, 9-octadecenamide Monopalmitic acid, oleic acid and tri-oleic triglycerides	(Anwar *et al.*, 2007), (Kumar *et al.*, 2012), (Gupta *et al.*, 2012), (Tumer *et al.*, 2015), (Soltan *et al.*, 2017), (Gupta *et al.*, 2018).
Antitumor activity	Leaves Seeds Flowers	Quercetin, kaempferol, niaziminin, niazimicin and more. 3-O-6x-oleoyl-β-D-glucopyranosyl-β-sitosterol, β-sitosterol-3-O-β-D-glu- copyranoside, 4-(α-L-rhamnosyloxy) phenylacetonitrile, 4-hydro-xyphenylacetonitrile and 4-hydroxyphenyl-acetamide. Cis-9-hexadecenal, quinic acid, 3,5-dihydroxy-6-methyl-2,3-dihydro-4H-pyran-4-1, 9-octadece-namide, methyl octadecenoate.	(Anwar *et al.*, 2007), (Berkovich *et al.*, 2013), (Soltan *et al.*, 2017), (Gupta *et al.*, 2018).
Antimicrobial activity	Leaves Seeds	Glucosinolates, rhamnose, pterygospermin, and isothiocyanates. 4-(4'-O-acetyl-a-Lrhamnopyranosyloxy) benzyl isothiocyanate. Griseofulvin, dechlorogriseofulvin, 8-dihydroramulosin and mullein.	(Shah *et al.*, 2016), (Soltan *et al.*, 2017), (Gupta *et al.*, 2018).
Anti-inflammatory activity		4-[(α-L- rhamnosyloxy)benzyl] isothiocyanate, 4-[(4′-O-acetyl-α-L-rhamnosyl-oxy)benzyl]isothiocyanate, quercetin, kaempferol glucosides, , 4-(2-O-acetyl- α-L-rhamnosyloxy)benzyl isothiocyanate, 4- (3-O-acetyl-α-rhamnosyloxy)benzyl isothiocyanate, 3,5-dihydroxy-6-methyl-2,3-dihydro-4H-pyran-4-1, 9-octadece- namide, aurantiamide acetate and 1,3-dibenzyl urea.	(Mahajan and Mehta, 2007), (Sulaiman *et al.*, 2008), (Coppin *et al.*, 2013), (Soltan *et al.*, 2017), (Gupta *et al.*, 2018).
Cardio-protective activity	Leaves	Niazinin, niazimicin, niaziminin, niazimin, niazirin, niazicin, niazir- inin, 4-(4′-O-acetyl-α-rhamnosyloxy) benzyl isothiocyanate. Glucomoringine had a hypertensive effect. Gossypetin, quercetagenin, and proanthocyanidins.	(Farooq *et al.*, 2012), (Kumar *et al.*, 2012), (Soltan *et al.*, 2017), (Gupta *et al.*, 2018).

Prominent phytochemicals responsible for antioxidant activity are present in all parts of the *M. oleifera* tree. These phytochemicals include vitamin C (ascorbic acid), vitamin A (ß-carotene), quercetin, kaempferol and phenolic acids (Kumar *et al.*, 2012). Extracts from roots and seed pods were also shown to exhibit antioxidant activities (Gupta *et al.*, 2012).

Leaf extracts have been found to show different activities like anticancer, cytotoxic, antileukemia, chemoprotective and antiproliferative properties (Berkovich *et al.*, 2013). These activities are due to certain bioactive chemicals, namely: quercetin, kaempferol, niaziminin and niazimicin (Soltan *et al.*, 2017).

Extracts processed from stem and seeds also show activities like anticancer, antitumor, and cytotoxic properties. These are merited to bio-active molecules, namely: 3-O-6x-oleoyl-β-D-glucopyranosyl-β-sitosterol, β-sitosterol-3-O-β-D-glucopyranoside, 4-(α-L-rhamnosyloxy)

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phenylacetonitrile, 4-hydro-xyphenylacetonitrile and 4-hydroxyphenyl-acetamide (Gupta *et al.*, 2018). The flowers of *M. oleifera* also exhibit antitumor and anticancer activities. Phytochemical analysis revealed bio-active molecules that are responsible for these activities, namely: cis-9-hexadecenal, quinic acid, 3,5-dihydroxy-6-methyl-2,3-dihydro-4H-pyran-4-1, 9-octadece-namide, methyl octadecenoate (Anwar *et al.*, 2007).

Leaf extracts of *M. oleifera* exhibited different inhibition levels against different bacteria, including: *Bacillus cereus*, *Bacillus subtilis*, *Staphylococcus aureus*, *Sarcinalutea* sp., *Escherichia coli*, acid fast *Mycobacterium phlei*, *Enterobacter aerogenes*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Providencia stuartii* (Gupta *et al*,. 2018). The bioactive components responsible for these inhibition patterns include glucosinolates, rhamnose, pterygospermin, and isothiocyanates. 4-(4'-O-acetyl-a-Lrhamnopyranosyloxy) benzyl isothiocyanate (Shah *et al.*, 2016).

Pods and flowers from *M. oleifera* had specific inhibition activity against three strains of *Vibrio*, namely *V. cholerae*, *V. vulnificus* and *V mimicus* (Brilhante *et al.*, 2015). *M. oleifera* inhibited both Gram-positive and Gram-negative bacteria, including *Staphylococcus aureus*, *Escherichia coli*, *Salmonella gallinarum* and *Pseudomonas aeruginosa* (Wang *et al.*, 2016).

Moringa oleifera seed extracts displayed high antifungal activity against *Trichophyton rubrum*, *T. mentagrophytes*, *Epidermophyton floccosum* and *Microsporum canis* (Soltan *et al.*, 2017). The antifungal components responsible for thisinclude griseofulvin, dechlorogriseofulvin, 8-dihydroramulosin and mullein. The seed extracts also had antiviral properties against HSV-1 and flowers were active against the Epstein Barr Virus (EBV), HIV and HSV-1 (Gupta *et al.*, 2018).

Potent anti-inflammatory agents identified in *M. oleifera* are 4-[(α-L- rhamnosyloxy)benzyl] isothiocyanate, 4-[(4′-O-acetyl-α-L-rhamnosyloxy)benzyl]isothiocyanate, quercetin, kaempferol glucosides, 4-(2-O-acetyl- α-L-rhamnosyloxy)benzyl isothiocyanate, 4- (3-O-acetyl-α-rhamnosyloxy)benzyl isothiocyanate, 3,5-dihydroxy-6-methyl-2,3-dihydro-4H-pyran-4-1, 9-octadece- namide, aurantiamide acetate and 1,3-dibenzyl urea (Gupta *et al.*, 2018; Coppin *et al.*, 2013). These agents are found in different parts of *M. oleifera*. It has also been stated that the anti-inflammatory response is due to the disruption of inflammation signaling pathways (Sulaiman *et al.*, 2008).

The leaves of *M. oleifera* exhibit hypotensive and bradycardiac activities. Through phytochemical analysis, it was discovered that niazinin, niazimicin, niaziminin, niazimin, niazirin,

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niazicin, niazirinin, 4-(4′-O-acetyl-α-rhamnosyloxy) and benzyl isothiocyanate were responsible for these activities while in contrast, glucomoringine had ans opposite hypertensive effect (Farooq *et al.*, 2012).

Leaves of *M. oleifera* were also reported to exhibit a positive effect on the circulatory system, potentially lowering mortality and morbidity in humans caused by coronary heart disease (Kumar *et al.*, 2012). This is due to bio-active chemicals present, namely gossypetin, quercetagenin, and proanthocyanidins.

2.1.2 Nutritional review of *Moringa oleifera*

Moringa oleifera has generated great interest as an animal feed because of its high nutritional content and low antinutrient composition. The examination of the nutritional composition of *M. oleifera* leaves revealed that they contain significant levels of proteins, minerals, vitamins, and other secondary metabolites. This is illustrated in Table 2.2 and compared to other forages.

Moringa oleifera leaves have a crude protein level that ranges from 23.0 to 30.3 % on a dry matter basis, which is greater than that of alfalfa meal, soybean meal and *Morus alba* (Table 2.2). *Moringa oleifera* leaf crude fibre content is just 5.9% overall, which is nearly equal to that of soybean meal, a common feed ingredient (Table 2.2). Good animal palatability is indicated by a low level of crude fibre (Su and Chen, 2020).

Table 2. 2: Nutritional value of *Moringa oleifera* versus other plant species used in livestock production (DM basis).

Feedstuff category	Plant species	Crude Protein	Crude lipid	Crude fibre	Ash
Woody plant forages	*M. oleifera*	23.0-30.3	7.09	5.9	7.6-12
	Morus alba	21.2-29.8	5.5	6.9	11.6
	Broussonetia papyrifera	21.0	3.2	9.1	12.1
	Caragana korshinskii	9.9	3.2	34.4	6.7
Conventional crop forages	Alfalfa meal	19.1	2.3	22.7	7.6
	Soybean meal	25.5	17.3	4.3	4.2
	Maize meal	9.4	3.1	1.2	1.2

Adapted and sourced from Su and Chen, (2020).

Table 2.3 illustrates the differences between seed oil meal, fresh leaves and dried leaves of *M. oleifera.* The crude protein (CP) and crude fibre (CF) levels between dried and fresh leaves do not differ significantly, with the CP levels being 26.8% and 24.3% respectively, and CF levels being 13.6 % and 12.2 % respectively. In both circumstances, values for the fresh leaves are relatively higher. This accounts for the neutral detergent fibre (NDF) and acid detergent fibre (ADF) levels, with the lignin levels being predominantly higher (7.0% vs 3.4%). The gross energy value of *M. oleifera* oil meal is higher than that of fresh leaves and dried leaves, with oil meal

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having 19.4 MJ/kg DM versus fresh – and dried leaves having gross energy levels of 18.6 ML/kg DM and 18.8 MJ/kg DM, respectively.

Table 2. 3: Main Component Analysis of different *Moringa oleifera* plant parts

Components	Unit	Oil meal	Leaves, Fresh	Leaves, Dried
Dry matter	% As fed	60.3	26.2	91.2
Crude protein	% DM	-	24.3	26.8
Crude fibre	% DM	-	13.6	12.2
NDF	% DM	9.4	28.3	22.7
ADF	% DM	6.7	19.3	15.3
Lignin	% DM	2.5	7.0	3.4
Ether Extract	% DM	0.4	5.4	6.4
Ash	% DM	5.7	10.3	10.8
Total sugars	% DM	-	-	11.0
Gross Energy	MJ/kg DM	19.4	18.6	18.8

Adapted and Sourced from Huezé *et al.* (2019)

Tables 2.4 and 2.5 illustrate the significant amino acid content of *M. oleifera,* which has been shown to be able to serve as a primary amino acid supplement for animal feeding when combined with other common forages (Su and Chen, 2020). Mune *et al.* (2016) reported that leaf powder contained a higher essential amino acid content than seed powder. In contrast, seed powder had a higher non-essential amino acid content than leaf powder, illustrated in Table 2.4. Mune *et al.* (2016) also reported that the limiting amino acids were cysteine and methionine for leaves and lysine for seeds.

Table 2. 4: Amino acid analysis of different *Moringa oleifera* plant parts.

Amino acids	Unit	Seeds	Leaves
Alanine	g/16g N	7.1	9.8
Arginine	g/16g N	5.9	4.3
Aspartic acid	g/16g N	8.0	9.8
Cystine	g/16g N	0.7	0.4
Glutamic acid	g/16g N	21.6	11.4
Glycine	g/16g N	13.0	10.6
Histidine*	g/16g N	2.7	2.0
Isoleucine*	g/16g N	3.5	5.0
Leucine*	g/16g N	7.2	9.7
Lysine*	g/16g N	1.6	5.5
Methionine*	g/16g N	1.4	1.4
Phenylalanine*	g/16g N	5.0	4.6
Proline	g/16g N	6.1	5.7
Serine	g/16g N	3.3	5.8
Threonine*	g/16g N	4.1	5.3
Tryptophan*	g/16g N	/	/
Tyrosine*	g/16g N	1.7	2.2
Valine*	g/16g N	7.1	6.7

Adapted and **sourced** from Mune *et al.* (2016).
*Essential amino acid

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Methionine, in contrast to lysine, is the first limiting amino acid in soybean meal (Farkhoy *et al.*, 2012). As shown in table 2.5 below, the Methionine level in *M. oleifera* leaf is only two-thirds that of soybean meal, greater than that of alfalfa meal and near the value of that of maize meal.

Table 2. 5: Comparison of amino acid content (in % gram) in *Moringa oleifera* leaves versus other forages.

Amino acid	Woody plant forage			Conventional crop forage		
	M. oleifera	*Morus alba*	*Broussonetia papyrifera*	Alfalfa meal	Soybean meal	Maize meal
Alanine	3.03	1.54	1.13	-	-	-
Arginine	1.78	1.80	1.00	0.78	2.53	0.38
Aspartic acid	1.43	3.06	1.88	-	-	-
Cystine	0.01	0.30	0.30	0.22	0.62	0.34
Glutamic acid	2.53	3.33	2.03	-	-	-
Glycine	1.53	1.57	1.06	-	-	-
Histidine*	0.72	0.69	0.42	0.39	1.10	0.23
Isoleucine*	1.18	1.43	0.89	0.68	1.57	0.26
Leucine*	1.96	1.35	1.69	1.20	2.75	-
Lysine*	1.64	1.80	1.25	0.82	2.43	0.22
Methionine*	0.41	0.52	0.36	0.21	0.60	0.43
Phenylalanine*	1.64	1.94	1.24	0.82	1.79	0.31
Proline	1.20	1.31	1.18	-	-	-
Serine	1.09	1.22	0.9	-	-	-
Threonine*	1.36	1.31	0.91	0.74	1.44	0.40
Tryptophan*	0.49	0.27	0.32	0.43	0.64	1.03
Tyrosine*	2.65	0.82	0.32	0.58	1.53	0.08
Valine*	1.41	1.76	1.40	0.91	1.70	0.26

Adapted and sourced from Su and Chen, (2020).

In a study done by Moyo *et al.* (2011), 17 fatty acids were identified, and 11 of these were identified as saturated fatty acids, but with low values (Table 2.6). The total poly-unsaturated fatty acid (PUFA) value was higher than that of the saturated fatty acids (SFA), with 52.21% vs 43.31%, respectively. This is a desirable characteristic (Hoffman and Wilklund, 2006), due to PUFA having the ability to decrease and control cholesterol levels and promote health of consumers and animals (Gopalakrishnan *et al.*, 2016). PUFAs are also the precursors of the long chain n-3 PUFAs in the biosynthesis of eicosanoids, which play a significant role as bioregulators for different cellular processes (Gopalakrishnan *et al.*, 2016). Thus, higher levels of PUFA, with lower levels of SFA are preferred by nutritionists and consumers due to their connected risk to cardio-vascular diseases and specific cancers (Alfaia *et al.*, 2009). The fatty acid content and composition in an animal's body are linked to the precursors in its diet, as certain fatty acids absorbed by the animal in the diet remain unchanged (Wood *et al.*, 2004).

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Table 2. 6: Fatty acid composition of dried *Moringa oleifera* leaves

Fatty acid	Unit	Quantity
Ether Extract	mean +/- %	6.50
Capric (C10:0)	mean +/- %	0.07
Lauric (C12:0)	mean +/- %	0.58
Myristic (C14:0)	mean +/- %	3.66
Palmitic (C16:0)	mean +/- %	11.79
Palmitoleic (C16:1c9)	mean +/- %	0.17
Margaric (C17:0)	mean +/- %	3.19
Stearic acid (C18:0)	mean +/- %	2.13
Oleic (C18:1c7)	mean +/- %	3.96
Vaccenic (C18:1c7)	mean +/- %	0.36
Linoleic (C18;2c9, 12(n-6)	mean +/- %	7.44
α-Linolenic (C18:3c9,12,15(n-3)	mean +/- %	44.57
ß-Linolenic (C18:3c6, 9, 12 (n-6)	mean +/- %	0.20
Arachidic (C20:0)	mean +/- %	1.61
Heneicosanoic (C21:0)	mean +/- %	14.41
Behenic (C22:0)	mean +/- %	1.24
Tricosanoic (C23:0)	mean +/- %	0.66
Lignoceric (24:0)	mean +/- %	2.91
Total saturated fatty acids (SFA)	**mean +/- %**	**43.31**
Total mono-unsaturated fatty acids (MUFA)	**mean +/- %**	**4.48**
Total poly-unsaturated fatty acids (PUFA)	**mean +/- %**	**52.21**
Total omega-6 fatty acids (n-6)	**mean +/- %**	**7.64**
Total omega-3 fatty acids (n-3)	**mean +/- %**	**44.57**
PUFA: SFA	**mean +/- %**	**1.21**
n-6/n-3	**mean +/- %**	**0.17**
PUFA: MUFA	**mean +/- %**	**14.80**

Adapted and Sourced from Moyo *et al.*, 2011

Moringa oleifera dried leaf powder contains adequate amounts of ß-carotene and vitamin A as shown in Table 2.7. ß-Carotene is the most dominant precursor to vitamin A, and healthy ruminants can efficiently convert ß-carotene to Vitamin A within their body (Moyo *et al.*, 2011). Vitamin A plays a critical role in efficient vision, bone growth and immunity in ruminants, as well as maintaining proficient levels of plasma-iron (Thurber and Fahey, 2010). *Moringa oleifera* also has high levels of vitamin C, 220 mg/100 mg in fresh *M. oleifera* leaves, which aids in the absorption of iron in the animal's body (Anwar *et al.*, 2007).

Table 2. 7: Analysis of vitamins in different parts of *Moringa oleifera*

Vitamins	Leaves, Fresh (mg/100g)	Leaves, Dried (mg/100g)	Leaves, Powder (mg/100g)	Seeds (mg/100g)
Vitamin A – ß-carotene	6.8	-	16.3	0.11
Vitamin B1 - Thiamine	0.06	2.02	2.64	0.05
Vitamin B2 – Riboflavin	0.05	21.3	20.5	0.06
Vitamin B3 – Nicotinic acid	0.8	7.6	8.2	0.2
Vitamin C – Ascorbic acid	220	15.8	17.3	4.5±0.17
Vitamin E – Tocopherol acetate	448	10.8	113	751.67±4.41

Adapted and Sourced from Gopalakrishnan *et al.* (2016)

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It is of notable interest that *M. oleifera* possesses high deposits of mineral elements relative to other plants used in production (Shi *et al.*, 2018). Calcium is of most interest due to its high deposition levels in dried leaves, at 36.5 g/kg DM (Table 2.8). Calcium has major health benefits in animals, namely, formation and maintenance of bones and teeth, normal blood clotting and optimal nervous function (Moyo *et al.*, 2011). Even micro elements like iron (Fe) have high deposits in different *Moringa oleifera* parts, which is a frequently deficient characteristic in other plants. Iron is necessary for normal oxygen transport, which is functioned by haemoglobin and myoglobin in red blood cells (Kozat, 2007). *Moringa oleifera* also has functional amounts of copper (8.3 Mg/kg DM) which is known to aid in decreasing the load of *H. contortus* in goats (Burke and Miller, 2006).

Table 2. 8: Mineral analysis of different *Moringa oleifera* parts.

Minerals	**Unit**	**Leaves, Dried**
Macro-elements	**g/kg DM**	
Calcium	g/kg DM	36.5
Phosphorus	g/kg DM	3.0
Potassium	g/kg DM	15.0
Sodium	g/kg DM	1.6
Magnesium	g/kg DM	5.0
Sulphur	g/kg DM	6.3
Micro Elements	**Mg/kg DM**	
Manganese	Mg/kg DM	86.8
Zinc	Mg/kg DM	31.0
Copper	Mg/kg DM	8.3
Iron	Mg/kg DM	490
Selenium	Mg/kg DM	363.0
Boron	Mg/kg DM	49.9

Adapted and sourced from Moyo *et al.* (2011)

2.1.3 The effect of feeding *Moringa oleifera* on livestock growth

Production systems will be compelled to employ more conventional crops in animal feeds due to the rapidly growing need for protein in consumer diets worldwide, which is generated by livestock production (Su and Chen, 2020). To support high levels of consumption, a corresponding elevation in animal feed production must be established. This can be done by supplementing an alternative protein feedstuff production (Su and Chen, 2020).

In the case of small ruminants, sheep provide animal protein that forms part of a balanced diet (Allam *et al.*, 2015). The largest problem that small ruminant production systems face is the inadequate supply of feed, both in quantity and quality of protein and micronutrient levels, especially during seasons of dry climatic conditions. *Moringa oleifera* possesses the nutritional value to play a key role in this shift towards alternative complementary feed sources. Table 2.9 summarises different studies conducted to improve the overall production of small ruminant livestock in intensive production systems.

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Table 2. 9: Studies performed using different *Moringa oleifera* plant parts as supplementation to conventional feedstuffs to increase overall production.

Reference	Moringa Source	Productional effect	
(Babiker *et al.*, 2017) n = 80	Feeding *M. oleifera* as partial replacement for alfalfa hay in ewes and goats.	Positive	P ≤ 0.05
(Allam *et al.*, 2015) n = 20	Use of *M. oleifera* leaves in fattening lamb rations.	Positive	NS
(Mahmoud, 2013) n = 21	Effect of feeding *M. oleifera* stems on productive performance of growing lambs.	Positive	P < 0.05
(Yusuf *et al.*, 2016) n = 18	Influence of Moringa leaf meal (MOLM) supplementation on growth performance, haematological parameters, and faecal egg count of West African dwarf sheep.	Positive	P < 0.05

2.1.4 *Moringa oleifera* fed as a feed additive

A feed component or collection of feed molecules that induces a desired animal response in a non-nutrient role is referred to as a feed additive (Hutjens, 1991). Natural additives in animal feed can increase the productivity and efficiency of animals by promoting digestibility through establishing a healthy bacterial population in the gastrointestinal tract (GIT) (Franz *et al.*, 2010).

A considerable amount of anthropogenic greenhouse gas is produced by the ever-expanding ruminant livestock sector. A ruminant's digestive tract produces methane (CH_4) as microbes decompose and ferment food in the rumen, causing it to be belched into the air (Su and Chen, 2020). A FAO report published in 2013 claims that the generation of carbon dioxide (CO_2) and methane (CH_4) are both largely accounted for by the livestock industry, which produces about 18% and 9%, respectively. In the stomachs of ruminant animals, methanogenic microbes (of the Archaea) generate methane. Biological and chemical CH_4 inhibitors have recently been added to animal feed as part of ruminant CH_4 reduction techniques to eliminate or at least restrict the activity of the methanogenic microbes in the gut (Haque, 2018). The methanogen-inhibiting properties of *M. oleifera* leaves led to suggestions that *M. oleifera* could be used as a substitute for essential antibiotic feed additives in order to upregulate ruminal fermentation pathways (Soliva *et al.*, 2005).

As natural substitutes for monensin in sheep diets, Soltan *et al.* (2018) tested the dosage (10-, 100-, and 1000 g/500mg dry matter (DM) diet) response effects of ethanolic extracts of *M. oleifera* leaves and root bark. The study drew the conclusion that *M. oleifera* leaves and root bark could be used as an effective natural intervention to monensin in sheep diets, not only to reduce CH_4 production but also to improve the ruminal efficiency of dietary nutrient use. In the extracts of *M. oleifera* leaves (all doses) and root bark at levels of 10 and 1000 g/500 mg DM, rumen CH_4 levels showed comparable decrease in levels to that resulting from monensin supplementation.

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It has also been demonstrated that the *M. oleifera* oil cake that remains after oil extraction retains the active elements for the coagulation of various undesirable molecules, making it a saleable good (Folkard *et al.*, 2001). The proteins in the above-mentioned oil cake have the ability to alter rumen fermentation due to their high positive charge (Folkard *et al.*, 2001) and antimicrobial impact (Makkar *et al.*, 2007). These proteins can also increase the post-ruminal protein supply because they have been demonstrated to slow down feed protein degradation in an *in vitro* rumen system (Hoffmann *et al.*, 2003).

A study was done by Salem and Makkar (2008), with the goal of assessing the effectiveness of adding defatted *M. oleifera* seed meal (DMOSM) to sheep diets. The research study used 24 Barbarine lambs divided into four groups, and the effects of DMOSM on feed intake, diet digestibility, microbial nitrogen supply, blood metabolites, and growth of lambs fed an oat-vetch hay based diet was assessed. All lambs received 100 g of soybean meal (SBM) mixed with 0 (control), 2 (low), 4 (mid), or 6 (high) g/dry matter (DM) of DMOSM each day in addition to unlimited amounts of hay. Lambs gained weight at rates of 63.8, 88.5, 97.0, and 76.6 g/day, respectively (Q; P=0.076), perhaps as a result of a trend toward increased microbial nitrogen supply (Q; P=0.109) and a trend toward higher N retention with higher levels of DMOSM in the diet (Q; P=0.086). Better nitrogen retention and microbial nitrogen efficiency at this level contributed to this rise (Delve *et al.*, 2001).

According to the study, lamb diets supplemented with DMOSM gradually showed a quadratic effect on average daily gain, with growth peaking at 4 g/day DMOSM given. Faeces excreted nitrogen instead of urine, which improved crop-livestock systems and decreased nitrogen leaching into groundwater. Additionally, lambs demonstrated greater plasma glucose levels, indicating improved energy value and better feed efficiency (Annison *et al.*, 2002). This might have been caused by enhanced rumen fermentation and feed protein protection (Hoffmann *et al.*, 2003). Nevertheless, daily gain dropped at 6 (high) g/day, perhaps as a result of an overreaction to antimicrobials causing an inefficient microbiome population, affecting the digestion of feed intake (Makkar and Becker, 1997; Makkar *et al.*, 2007).

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2.2 A review of *Haemonchus contortus*

Zarlenga *et al.* (2016) stated that *Haemonchus contortus* has been acknowledged and studied for the past 2 decades in terms of its economic and veterinary importance. The study further explains that *H. contortus* is one of 12 confirmed species of *Haemonchus* and identified based on their abomasal profile in sheep to date.

Haemonchus contortus is regarded as the single most significant hindrance to sheep production in South Africa (Vatta *et al.* 2001) due to it causing the parasitic gastroenteritis (PGE) complex, a disease entity (Adamu *et al.*, 2013) which is capable of acute disease and increased mortality rates (Allonby and Urquhart, 1975). According to Ademola and Eloff (2011), helminthosis is a significant factor in sheep mortality and decreased productivity, which can result in mortality levels between 10-20% in production enterprises (Ilha *et al.*, 2005), with *H. contortus* infections causing 20-60% weight loss in sheep, affecting younger animals more (Qamar *et al.*, 2011). These production losses can be divided into direct and indirect losses. Direct losses are due to a drop in production, namely carcass quality and weight, while indirect losses are caused by the cost of drugs, labour and equipment cost used for drenching in control strategies (Soulsby, 1986; Kassai, 1999).

Other factors relating to losses result from the increasing resistance in *H. contortus*, with the first reported case in South Africa being in 1975 for the anthelmintic group benzimidazole (Berger, 1975). This emergence is mostly due to the uncontrolled and excessive dosage amounts of anthelmintic drugs (Adamu *et al.*, 2013). Subsequently, other anthelmintic groups were shown to be susceptible to *H. contortus* due to resistance being developed against the anthelmintic (Malan *et al.*, 1990; Van Wyk *et al.*, 1997).

With this emergence in resistance, a need for certain alternatives to combat the resistance was required. One such option was through vaccine development but was rendered unsuccessful due to the complexity of the antigenic sequence in *H. contortus* (Le Jambre *et al.*, 2008). Another option was through nematode trapping fungi, which were used as a treatment mechanism for pastures but were unsuccessful due to the complexity of the mechanism (Grønvold *et al.*, 1993; Waller and Faedo, 1996).

Indigenous plants seem to be a viable option at this time due to the phytochemical balance in certain medicinal plants which might be able to combat intestinal nematodes as well as decrease the effects of resistance. It is also a much more sustainable method, due to plants being more readily available for rural development (Hammond *et al.*, 1997; Danø and Bøgh,

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1999). A key issue is the limited validation of alternative plant species for their effectiveness in this area (Maphosa *et al.*, 2010).

2.2.1 Physiology and Epidimology

Haemonchus contortus is a nematode parasite of the Phylum: Nematoda, Class: Secernentea, Order: Strongylida, and Family: Trichonstrongylidae (Flay *et al.*, 2022). *Haemonchus contortus* is also known as the “barber’s pole worm” and the “wire worm”. It is a blood-sucking, abomasum nematode where the mature larvae can remove approximately 30 µl of blood daily, which can cause a rapid anaemic response and even death (Emery *et al.*, 2016). It is stated that sheep that are infected by the *H. contortus* will lose digestive capabilities which affects the uptake of protein, certain organic matter, and energy particles (Rowe *et al.*, 1982).

Haemonchus contortus has a short life cycle of roughly 20 days and the female mature worm can lay between 5 000 and 15 000 eggs daily (Emery *et al.*, 2016). This, coupled with the fact that it has an establishment rate of 60% (Dineen *et al.*, 1965), confirms that *H. contortus* is one of the most negative influential nematodes in livestock production. Another factor adding to the negative influence is that *H. contortus* has evolved to adapt to specific environmental niches and may use more than one host to survive and complete their life cycle (Sallé *et al.*, 2019). Internal parasites in general have complicated cycles of life which include intermediate hosts, vectors, and time spent in the external environment, where they must endure severe and unpredictable circumstances like cold or dry conditions in between infections of their hosts (O’Connor *et al.*, 2006).

Figure 2.3 shows the life cycle of *H. contortus*, spanning pre– to post infection of the sheep. The life cycles can be divided into two stages, namely the free-living stage (eggs to L1 to L3) and the parasitic stage (L4 to L5) (Adduci *et al.*, 2022). It has been stated that stage 3 free living larvae (L3) show a high survivability rate in areas where the environment has a high humidity and warm temperature (Adduci *et al.*, 2022). Gibbs (1986) suggests that the changes in environmental conditions, either in temperature or humidity, are the main reasons for L4 larvae to undergo hypobiosis, which is a state of reduced metabolic activity.

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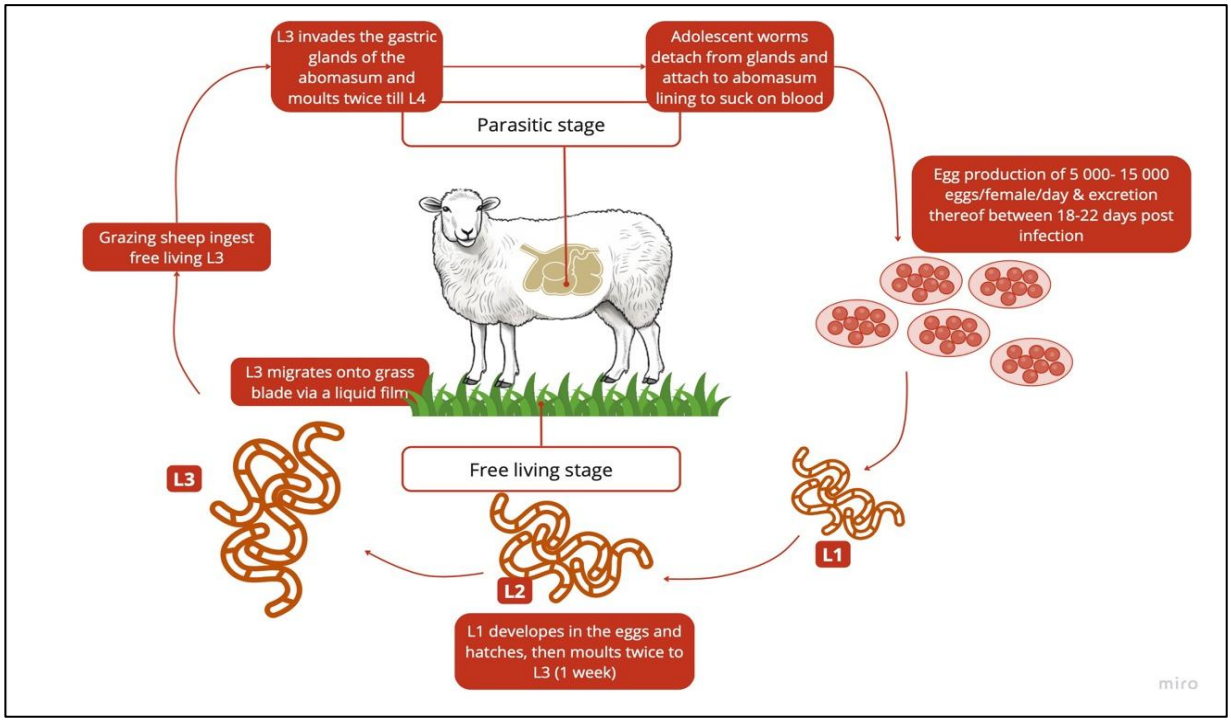

Figure 2. 3: Life cycle of *H. contortus* (Adapted from Adduci *et al.*, 2022)

2.2.2 Symptoms of haemonchosis and effects on small-stock production

Animals affected by haemonchosis (infection due to *H. contortus*) show signs of anaemia, eosinophilia and hypoproteinaemia (Gareh *et al.*, 2021). This occurs more in young sheep with weaker immune systems (i.e. during their first grazing season) as well as in adult sheep with weak immune systems (Zajac, 2006). The pathophysiology of haemonchosis is predominantly focussed on hypoalbuminemia and anaemia, with the intensity depending on the parasitic load, stage of development, the weight and age of the infected animal and finally the response to the infection by the host (Flay *et al.*, 2022).

Haemonchosis can be classified into three stages of infection, namely acute, hyperacute and chronic (Besier *et al.*, 2016). According to Besier *et al.* (2016), acute haemonchosis is depicted as a larval burden of 2 000 – 20 000 larvae per animal, whereas hyperacute haemonchosis shows more severe levels of infection, ranging up to 30 000 larvae per animal. The amount of blood lost may be up to half that of the lamb, and the assumption of pathogenic death may be based on the lamb's hypovolemic shock (Flay *et al.*, 2022).

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Acute haemonchosis occurs over a longer period with lower blood loss, causing hypoalbuminemia due to a reduction in the intravascular oncotic osmotic pressure and oedema which can be confined to the intermandibular space, referred to as “bottle jaw”, and the cervical region (Naeem *et al.*, 2021). Chronic (or subclinical) haemonchosis is defined by Besier *et al.* (2016) as the low ingestion of free living L3 larvae of *H. contortus* to a point where the infections may not be diagnosed. This is usually due to the environment not being suitable for the free living L3 to survive outside of the host. The infection can be identified by decreased production, feed conversion, lower milk yield of lactating ewes and overall growth impairment (Cobon and O’Sullivan, 1992).

2.2.3 Resistance and the effect on livestock production

One crucial threat to small stock production systems is the capability of *H. contortus* to establish resistance against nearly every class of anthelmintics, as well as combinations thereof (Lyndal-Murphy *et al.*, 2014). The definition of the term resistance in practical terms relating to *H. contortus* is stated by Kotze and Prichard, (2016) as a shift in an organism's reaction to a drug's treatment such that its efficacy is lower than it was when it was first made available for usage in the field. This is in accordance with Coles *et al.* (1992), who defined resistance criteria with the standards established by the World Association for the Advancement of Veterinary Parasitology (WAAVP), which deemed resistance to exist in the test population if the percentage reduction in egg count after drug treatment is 95%, and the lower 95% confidence level is 90%, as measured in a faecal egg count reduction test (FECRT).

It is reported that *H. contortus* has fallen under the resistance definition stated above, meaning that the efficacy levels of numerous anthelmintic drugs are below 95% (Kotze and Prichard, 2016). The only defence against the resistance of *H. contortus* in today’s industry is aided by the fact that the resistance to the different mode of actions by different anthelmintic drug classes vary. Thus, a combination of different anthelmintic drugs from different drug classes can be administered, and this process can be used for the time being to control the infection rate of *H. contortus* until the efficacy levels are zero (Bartram *et al.*, 2012).
Table 2.10 illustrates the different anthelmintic drugs that are considered to fall under the resistance factor of *H. contortus*, showing the adaptation of *H. contortus* to the mode of action of each drug.

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2.2.4 Diagnosis of Haemonchosis and anthelmintic resistance

The first method to be considered into detecting *H. contortus* infection is through a process called FAMACHA® (FAffa MAlan CHArt). This method forms a strategy where certain individual animals can be investigated for an infection and treated individually rather than as a whole group (Van Wyk *et al.*, 2006). Operators investigate the severity of infection by checking the conjunctival colour of the eyelid to assess the anaemia level of the individual animal and select these animals for specific treatments to that correlating level (Mphahlele *et al.*, 2019). This is expressed in a score of 1 – 5, increasing from normal to an utmost terminal situation, where 1 is reflected as a red/pink colour and 5 reflected as white (Van Wyk and Bath, 2002). The FAMACHA method has provided an excellent practical approach in assessing haemonchosis in animals, with a crucial benefit being that specific animals with a more severe infection can be identified and treated to prevent large production losses as well as lowering grassland contamination as these animals produce large numbers of eggs (Mphahlele *et al.*, 2019).

Table 2. 10: Recorded number of anthelmintic drugs against which *Haemonchus contortus* is resistant.

Country	Drug class	Drug Resistance	Reference
South Africa	Benzimidazoles	Thiabendazoles	Berger (1975)
		Parbendazole	Berger (1975)
		Fenbendazole	Berger (1975)
		Mebendazole	Berger (1975)
	Salicylanilides	Rafoxanide	Van Wyk and Gerber (1980)
		Closantel	Van Wyk *et al.* (1982)
	Macrocyclic lactones	Ivermectin	Craig and Miller (1990)
Australia	Benzimidazoles	Thiabendazoles	Le Jambre *et al.* (1976)
		Fenbendazole	Hogarth-Scott *et al.* (1976)
		Albendazole	Gunawan *et al.* (1979)
		Oxfendazole	Guinan and Kieran (1980)
		Mebendazole	Hall *et al.* (1978)
	Salicylanilides	Closantel	Rolfe *et al.* (1990)
	Organophophates	Naphthalophos	Green *et al.* (1981)
	Imidazothiazoles	Levamisole	Green *et al.* (1981)
	Macrocyclic lactones	Ivermectin	Le Jambre (1993)
		Abamectin	Wooster *et al.* (2001)
		Moxidectin	Love *et al.* (2003)
United States of America	Benzimidazoles	Thiabendazoles	Drudge *et al.* (1964)
	Macrocyclic lactones	Ivermectin	Craig and Miller (1990)
		Doramectin	Terrill *et al.* (2001)
Kenya	Benzimidazoles	Fenbendazole	Mwamachi *et al.* (1995)
	Macrocyclic lactones	Ivermectin	
Zambia	Benzimidazoles	Albendazole	Gabriël *et al.* (2001)
	Macrocyclic lactones	Ivermectin	
Zimbabwe	Benzimidazoles	Fenbendazole	Mukaratirwa *et al.* (1997)
		Albendazole	Matika *et al.* (2003)
		Oxfendazole	Boersema and Pandey (1997)
	Imidazothiazoles	Levamisole	
	Salicylanilides	Rafoxanide	

Adapted and sourced from Mphahlele *et al.* (2019) and Kotze and Prichard (2016).

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2.2.5 Monitoring systems for resistance

Kotze and Prichard (2016) stated that the monitoring systems in place to detect resistance can be divided into three sections, namely *in vivo* resistance test, *in vitro* bioassay-based test, and molecular based tests. The most well known and most used *in vivo* test used to detect resistance in *H. contortus* in sheep is the faecal egg count reduction test (FECRT), this is done by measuring the differences between pre – and post treatment egg counts (Kotze and Prichard, 2016). Even though the FECRT is suggested by the World Association for the Advancement of Veterinary Parasitology (WAAVP), it still has a few limitations, namely cost of administering the test, inadequate accuracy due to the unspecific techniques in counting the eggs, and difference in egg output (Martin *et al.*, 1989; Waller *et al.*, 1989; McKenna, 1990; Taylor *et al.*, 2002; Miller *et al.*, 2006; Levecke *et al.*, 2012; Kotze and Prichard, 2016).

The egg hatch assay and larval development assay falls under the *in vitro* bioassay- based tests. Le Jambre (1976) first reported that the egg hatch assay (EHA) only records the resistance factor for the drug class benzimidazoles. The EHA process is defined as diluting the eggs in an increasing concentration interval in a drug classed under benzimidazole or it can be diluted into a “discriminating dose”, meaning the dose needed to hinder 99% of egg hatching, and incubated for 48 hours. The eggs that hatch are counted using an inverted microscope (Mphahlele *et al.*, 2019). This test can detect resistance from the eggs at a range of 2-3% (Lindberg and Vatta, 2006). Von Samson-Himmelstjerna *et al.* (2009) performed critical tests in the pursuit of standardizing the detection of resistance in the benzimidazole class of drugs, publishing a standardised manual that describes the methods and explain the results thereof.

Several assays have been developed and published regarding the Larval Motility Assay (LMA) (Kotze and Prichard, 2016). For this study, the focus was on the assay published by Gill *et al.* (1991) as the basic principles towards the overall study were the determination of whether a larva is mobile or immobile following drug exposure.

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2.3 Anthelmintic potential of different medicinal plants against *Haemonchus contortus*

An important percentage of pharmaceutical research and development in animal health has been devoted to the discovery of anthelmintics, and this area of study may be the only one where success rates for animal health surpass those for human health (McKellar and Jackson, 2004). There are 50,000–70,000 plant species that have applications in both traditional and contemporary medicine (Newman and Cragg, 2012). Thus, plants used for medicinal purposes are still an important aspect in phytochemical studies due to being readily used in traditional medicine, which can hold great promise for potential anthelmintic studies (Aremu *et al.*, 2012). Certain secondary metabolites found in plants are bioactive with a wide range of effectiveness against multiple microorganisms including parasites. Some of these compounds are shown in Table 2.11.

Table 2.11: Anthelmintic bioactive compounds and class found in certain plant species with their respective molecular structure.

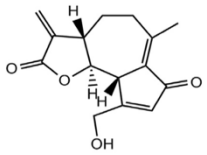
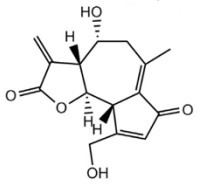
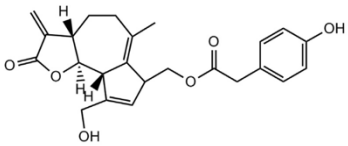
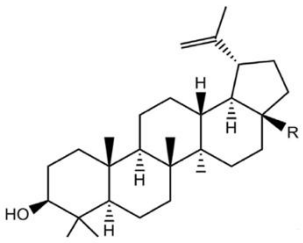
Plant Species	Name/class of Compound	Molecular Structure	References
Cichorium intybus	8 – deoxylactucin **Guaiane sesquiterpenoids**		Foster *et al.* (2011)
	Lactucin **Guaiane sesquiterpenoids**		
	Lactucopicrin **Guaiane sesquiterpenoids**		
Curtisia dentata	Lupeol R:CH_3 **Lupane triterpenoids**		Shai *et al.* (2009)
	Betulinic acid R: COOH **Lupane triterpenoids**		

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Table 2.11: Anthelmintic bioactive compounds and class found in certain plant species with their respective molecular structure.

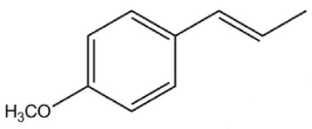
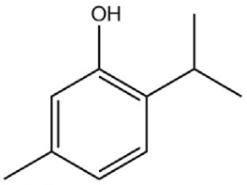
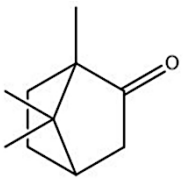
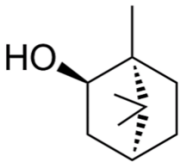
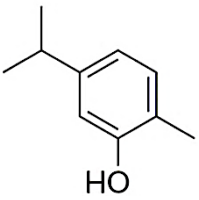
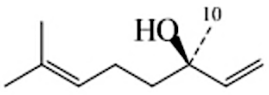
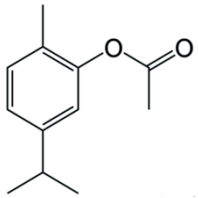
Plant Species	Name/class of Compound	Molecular Structure	References
Lippia sidoides	Anethole **C - Cinnamic acids** and **derivatives** **P - Shikimates** and **Phenylpropanoids**		Camurça-Vasconcelos *et al.* (2007)
	D – Pinitol **C - Cyclitols**		
Artemisia lancea	1,8 – Cineole **C - Monocyclic monoterpenoids**		Zhu *et al.* (2013)
	Camphor **C - Camphane monoterpenoids**		
Zanthoxylum bungeanum.	Borneol **C - Camphane monoterpenoids**		Qi *et al.* (2015)
Arisaema franchetianum	Carvacrol **C - Menthane monoterpenoids**		Zhu *et al.* (2013)
	Linalool **C - Acyclic monoterpenoids**		
NA	Carvacryl acetate **C - Menthane monoterpenoids**		André *et al.* (2020)

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Table 2.11: Anthelmintic bioactive compounds and class found in certain plant species with their respective molecular structure.

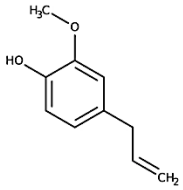
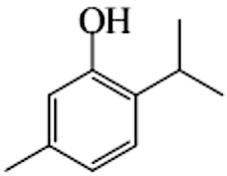
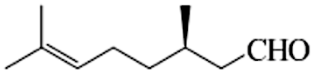
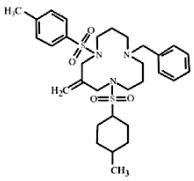
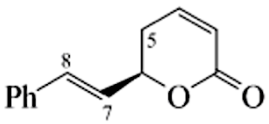
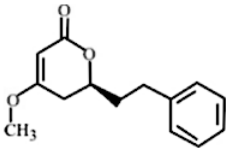
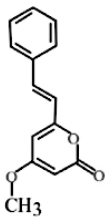
Plant Species	Name/class of Compound	Molecular Structure	References
Ocimum gratissimum L.	Eugenol **C - Cinnamic acids** and **derivatives** **P - Shikimates** and **Phenylpropanoids**		Pessoa *et al.* (2002)
Lippia origanoides	Thymol **C - Menthane monoterpenoids**		Katiki *et al.* (2019)
Myrtaceae[A]	Citronellal **C - Acyclic monoterpenoids**		Araújo-Filho *et al.* (2019)
Leguminosae[A]	Lectin **C – Flavonoids**		Silva *et al.* (2019)
Cryptocarya massoy	Goniothalamin **C - Kavalactones** and **derivatives** **P - Shikimates** and **Phenylpropanoids**		Herath *et al.* (2019)
Piper methysticum	Dihydrokavain **C - Kavalactones** and **derivatives** **P - Shikimates** and **Phenylpropanoids**		
	Desmethoxyyangonin **C - Kavalactones** and **derivatives** **P - Shikimates** and **Phenylpropanoids**		

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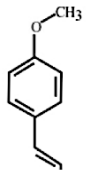
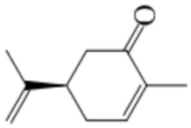
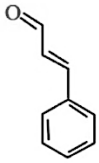
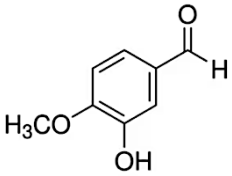
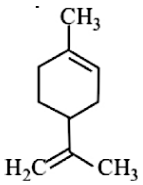
Table 2.11: Anthelmintic bioactive compounds and class found in certain plant species with their respective molecular structure.

Plant Species	Name/class of Compound	Molecular Structure	References
Piper methysticum	Yangonin **C - Kavalactones** and **derivatives** **P - Shikimates** and **Phenylpropanoids**		Herath *et al.* (2019)
Produced Synthetically	Carvone **C - Menthane monoterpenoids**		Katiki *et al.* (2019)
Produced Synthetically	Cinnamaldehyde **C - Cinnamic acids** and **derivatives** **P - Shikimates** and **Phenylpropanoids**		Katiki *et al.* (2017)
	Vanillin **C - Cinnamic acids** and **derivatives** **P - Shikimates** and **Phenylpropanoids**		
Produced from citrus peel	Limonene **C - Menthane monoterpenoids**		

Adapted and sourced from Ali *et al.* (2021), Romero-Benavides *et al.* (2017) and Aremu *et al.* (2012).
All chemical structures were sourced from references in table, Dictionary of Natural Products and Pubchem.com.
[A]family name of plant species.

2.4 Anthelmintic properties of *Moringa oleifera*

Plants and various components of them have been used to treat diseases since ancient times, according to scripted traditions and folklore, and this practice is known as "phytotherapy" today. (Fatima *et al.*, 2014). Among all diseases that may affect livestock production in tropical areas, intestinal parasites are the most destructive (Githiori *et al.*, 2004). If parasites infest a host, whether internal or external, it will result in stunted growth, a decrease in productivity, increase in morbidity and grave economic disturbance (Fatima *et al.*, 2014).

To combat helminth parasites in livestock, farmers spend a significant amount of money annually, relying on the frequent use of commercial anthelmintic medications. Nematodes have, however, evolved resistance to several anthelmintic groups (Tayo *et al.*, 2014). The potential of

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drug residues in animal products and toxicity from improper dose administration are two further significant issues linked to the usage of synthetic pharmaceuticals (Bachaya *et al.*, 2009). Additionally, *M. oleifera* is used to treat schistosomiasis, leishmaniasis, trypanosomiasis, dracunculiasis and filariasis, indicating that it naturally has antiparasitic properties (Fahey, 2005; Wang *et al.*, 2016).

According to Tayo *et al.* (2014), the infused and macerated aqueous and ethanolic leaf extracts showed varied degrees of ovicidal and larvicidal effects against the fresh and embryonated eggs as well as the L1 and L2 larvae of *H. contortus*. Salles *et al.* (2014) also deduced that the seed fraction of low molecular weight molecules (12 kDa) may contain chemicals that prevent over 90% of eggs from hatching. Cabardo Jr *et al.* (2017) performed a study where the ethanolic and aqueous seed extracts of *M. oleifera* were tested for their anthelmintic properties against *H.* contortus eggs and infective stage larvae (L3s) as recorded in Table 2.12. These studies found that both extracts prevented the development of larvae inside the eggs and immobilised the L3s. Therefore, plant bioactive substances with anthelmintic properties against the eggs and L3s of *H. contortus* are present in *M. oleifera* seed extracts.

2.5 *Moringa oleifera* as a potential anthelmintic against *Haemonchus contortus*

According to Ekor (2014), almost 80% of the world's population relies solely on plants for health and healing, and 25% of integrated pharmaceuticals are derived from medicinal and aromatic plants. As mentioned before, *M. oleifera* has long been used as an herbal medicinal product in both India and Africa due to the many phytochemicals present, making it a good medicinal agent. Specific parts of the *M. oleifera* plant are used for human and animal sustenance, as well as to treat disorders in many parts of Africa and Asia (Mbikay, 2012). Bioactive plant products have drawn a lot of attention since they are more socially and environmentally acceptable than their synthetic counterparts (Singh *et al.*, 1996).

Salles *et al.* (2014) conducted a study where the *M. oleifera* possessed multiple bioactive compounds and found that *M. oleifera* showcased an array of inhibitory effects against serine protease and cystine protease activity, as well as hemagglutinating and haemolysin activity. Many studies soon followed where the anthelmintic properties of *M. oleifera* was tested through different nematode assays which are summarised in Table 2.12.

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Table 2.12: Different studies showing the anthelmintic activity of different *Moringa oleifera* extracts.

Study	Species	Assay	Efficacy	Concentration	P-Value	Extract Used
Cabardo Jr and Portugaliza (2017)	*H. contortus*	EHA LMA 	>90% >80% >90% >55%	15.6mg/ml 15.6mg/ml 7.8mg/mL 7.8mg/ml	P > 0.05 P > 0.05 	Ethanolic Seed Aqueous Seed Aqueous Seed Ethanolic Seed
Tayo *et al.* (2014)	*H. contortus*	EHA L_1MA L_2MA	~99% 98.8% 100%	5mg/mL 5mg/mL 5mg/mL	P < 0.05	Ethanolic Leaf
Pedraza-Hernández *et al.* (2021)	*Trichostrongylus* sp.	FECRT	31 g/goF 0 g/goF 0 g/goF	0 mg/ml 7mg/ml 15mg/ml	P = 0.002	Hydroalcoholic - Leaf
Kandil *et al.* (2018)	*Fasciola hepatica*	EHA	96.9% @24hrs 100% @48hrs 100% @72hrs	50mg/mL 25mg/mL 10mg/mL	P < 0.05	Methanolic Seed

EHA: Egg Hatch Assay,
FECRT: Faecal Egg Count Reduction,
LMA: Larval Motility Assay,
L_1MA: Larval stage 1 Motility assay, L_2MA: Larval stage 2 Motility Assay.
g/goF: Gram of eggs per gram of faeces

These reported studies indicate the potential role *M. oleifera* can play in the battle against helminthosis and more specifically haemonchosis.

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Chapter 3: Materials and Methods

3.1 Introduction

An investigation was conducted to investigate the potential anthelmintic properties of *Moringa oleifera* against *Haemonchus contortus.* In this study, ethanolic extracts from *M. oleifera* seeds and leaves were tested. The extracts were fractionated, yielding five fractions for the seeds and seven fractions for the leaves, ranging from polar to non-polar. These fractions were tested on *H. contortus* eggs and larvae using an Egg Hatch Assay (EHA) and Larval Survivability Assay (LSA).

The anthelmintic potential (egg hatch inhibition, larval mortality or both) of the extracts and fractions was then evaluated using an *in vitro* assay. Eggs and larvae of *H. contortus* were gathered, and different concentrations of these were made. Mortality rates were noted after egg hatch and larval survivability assays were completed.

The approved ethical clearance number was NAS 356/2022.

3.2 Collection and preparation of plant materials

The leaf and seed materials were collected from a private *M. oleifera* cultivation farm at Inhassune Agricola, Inhassune, Panda District, Inhambane, Mozambique (24º14’13.1” S: 34º48’27.3” E) where it was air dried in the shade until crisp to touch. The dried *M. oleifera* leaves and seeds were packaged in a zip-lock bag and transported to the Biodiscovery Centre’s laboratory at the University of Pretoria, where it was milled into a fine powder with a Macsalab Mill Model 2000 LAB Eriez® and stored in a cold, dark and dry room.

3.3 Experimental design

3.3.1 *Haemonchus contortus* larvae and egg solution preparation

The *Haemonchus contortus* eggs were collected from the rectum of 12 infected sheep at Innovation Africa, University of Pretoria, following the methods of Hubert and Kerboeuf (1992). The faecal samples, weighing approximately 10-15 grams, were placed in zip-lock bags and transported in an ice-filled cooler to prevent any egg or larval development. A 2-gram sample of faeces was mixed with water to create a slurry, which was then sieved through a 25 µm sieve to remove large particles. The resulting solution was combined with a sugar water solution (1:25

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ratio) and allowed to stand for 15 minutes, enabling the eggs to float to the surface. A small aliquot was taken for counting using a McMaster slide to establish baseline egg numbers per gram. After filtering the solution through 100 µm and 63 µm sieves, the top layer of the solution was transferred to a centrifuge tube and stored at 5-10ºC for analysis.

3.3.2 Extraction and fractionation

Crude extracts were prepared by macerating the powdered *M. oleifera* leaves and seeds separately in ethanol for 72 hours. The mixtures were then filtered through Whatman No. 1 filter paper to obtain the *M. oleifera* leaf and seed crude extracts. Following this, the crude extracts underwent fractionation using a liquid-liquid partitioning method, resulting in seven distinct fractions for the leaves and five fractions for the seeds. Each *M. oleifera* leaf and seed crude extracts as well as fractions of each (7 and 5) were collected and concentrated into 3 different concentrations of 5-, 10- and 15 mg/ml.

3.3.3 Egg hatch assay (EHA) and larval survivability assay (LSA)

The experimental design was divided into two parts, namely the EHA and LSA of the *M. oleifera* leaves and the EHA and LSA of the *M. oleifera* seeds. This was done due to the number of treatment groups differing between the plant materials. A 2x10x3x4 factorial design was established for the *M. oleifera* leaf EHA and LSA, where both the assays had the crude extract (CE), fractions 1-7 (F1-F7) and two control groups (0.5% albendazole and ivermectin positive control group for EHA and LSA, respectively, and distilled water for the negative control group). Each of the CE and F1-F7 treatments were allocated to three concentration treatment groups, namely, 5, 10 and 15 mg/ml. Each of the treatment concentrations were replicated four times, with each replicate receiving 10-45 *H. contortus* unhatched eggs (EHA) and 10-25 stage 3 (L3) *H. contortus* larvae (LSA) allocated to each treatment group. Each replicate received 50µL of concentrated treatment solution groups of *M. oleifera* CE and seed and leaf fractions.

A 2x8x3x4 factorial design was established for the *M. oleifera* seed EHA and LSA, where both the assays had the crude extract (CE), fractions 1-5 (F1-F5) and two control groups (0.5% albendazole and ivermectin positive control group for EHA and LSA, respectively, and distilled water for the negative control group). Each of the CE and F1-F5 treatments were concentrated into three concentration treatment groups, namely, 5, 10 and 15 mg/ml. Each of the concentration treatment groups were replicated four times, with each replicate receiving 10-45 *H. contortus* unhatched eggs (EHA) and 10-25 stage 3 (L3) *H. contortus* larvae (LSA) allocated

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to each treatment group. Each replicate received 50µ L of concentrated treatment solution groups (CE and fractions thereof).

This resulted in 240 samples analysed for *M. oleifera* leaves and 192 samples analysed for *M. oleifera* seeds.

3.4 Nutritional composition

A proximate analysis was done by ChemNutri Analytical (PTY) Ltd on the air dried *M. oleifera* leaf and seed powders. The methods used were as follows:

- → Ash: AOAC method 942,05
- → Ether Extract (fat): AOAC method 920,3a
- → Crude Fibre: AOCS method Ba 6a-05
- → Crude Protein (Dumas): AOAC method 990,03
- → Moisture: ISO 6496

These results were used to investigate the differences between different plant species as well as between different *M. oleifera* cultivars.

3.5 Preparation of the *Moringa oleifera* ethanolic (Mo:EtOH) extracts

Preparation of the Mo:EtOH extracts followed the same methodologies done by Páez-León *et al.* (2022) and Invernizzi *et al.* (2022). Fifty grams of the leaf and seed powder were extracted using a glass extraction vessel, fitted with a PTFE (Polytetrafluoroethylene) stopcock and a sintered glass filter at a porosity level of 3. Ethanol was added and left to soak for 24 hours, followed by vacuum filtration through Whatman no. 1 filter paper. The filtrate was collected and stored, and the residue subjected to two repeats of the process. Fresh ethanol was used for each repeat. The three filtrates were combined, then concentrated *in vacuo* by rotary evaporation. Drying was accomplished at room temperature in a fume cupboard. Characterisation and fractionation of the extracts was done by UPLC-UV/QTOF/MS (Ultra-high pressure liquid chromatography – Ultra Violet/Quadrupole time-of-flight/Mass Spectrometry). Approximately 300 mg of each leaf and seed concentrate were subjected to fractionation by solid phase extraction (SPE) using a Gilson GX-241 ASPEC® liquid handler.

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3.6 Fractionation of ethanolic seed and leaf extracts of *Moringa oleifera*

The dry extract was fractionated using the same techniques described by Invernizzi *et al.* (2022). Using a Gilson GX-241 ASPEC® liquid handler equipped with a Gilson Verity® 4060 pump and TRILUTION® LH software, each extract (leaf and seed) was fractionated into seven and five different fractions using a 6 mL ThermoScientific® HyperSep™ C8 Solid Phase Extraction (SPE) cartridge (2 g sorbent). After dissolving the seed and leaf extracts in 5 millilitres of methanol (MeOH), it was adsorbed onto a cotton wool roll. After being dried in an SP Scientific® Genevac® HT-6 (Series 3i), the cotton wool roll, containing the absorbed extract, was transferred to an empty 6 mL SPE cartridge. Thereafter it was equilibrated with the first eluent, which was 19:1 H_20:MeOH. The SPE cartridge, carrying the extract, was connected on top of the C8 SPE cartridge. Seven and five fractions (leaf and seed) were produced, along a decreasing polarity gradient, by applying 9 mL of each of the following solvent systems (11 mL of first system) in sequence: H_2O:MeOH – 19:1, 4:1, 3:2, 2:3, 1:4, 100% MeOH, ACN:MeOH 1:1. Each fraction was individually collected. The fractions were dried as a collective in an SP Scientific® Genevac® HT-6 (Series 3i).

Throughout fractionation, de-ionised water (dH_2O) and ROMIL® SpS™-grade methanol 215, and ROMIL® SpS™-grade acetonitrile 200 was used for sample solubilisation and as mobile phase components (Invernizzi *et al.*, 2022).

3.7 UPLC-UV/QTOF/MS analysis of extracts

The UPLC-UV/QTOF/MS analysis followed the same methodologies discussed by Zhou *et al.* (2016) and were followed below. Of each extract a 5000 ppm solution was prepared with a 1:1 solvent system of ROMIL® SpS™-grade methanol 215 and dH_2O. The samples (5.000 µL each respectively) were chromatographically separated on a Waters® Aquity™ UPLC® BEH C18 (1.7 µm particle diam., 2.1 × 100 mm). Component separation was achieved with the following gradient solvent system (dH_2O(+0.1% Formic acid (99%)) : MeOH (+0.1 Formic acid (99%)) initial conditions: 97:3 (hold for 1.00 min), 100% MeOH (at 14.00 min, hold for 3 .00 min), 97:3 (at 17.50 min, hold for 2.50 min). The formic acid (99%) spiked methanol was prepared from ROMIL® UpS™-grade methanol.

Eluting components were detected with an Aquity™ PDA eλ Detector and a Xevo® G2 QTof mass analyser (resolution of 22500 FWHM). The detectors were set to the ranges 220-700 nm, and 50-1200 Da respectively. Mass accuracy was maintained by simultaneous periodic infusion of leucine enkaphalin ($m/z[M+H]^+$ = 556.2771, $m/z[M-H]^-$ = 554.2615) during runs.

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3.8 Extract and control solution preparation

The preparation of Mo:EtOH extracts and fractionation of the Mo:EtOH extracts yielded certain solution groups that were used in each assay named crude extract (CE) and fractions 1 to 7 (F1-F7) for *M. oleifera* leaves and fractions 1 to 5 for *M. oleifera* seeds. The reason *M. oleifera* seeds produced fewer fraction groups than *M. oleifera* leaves was due to the low volumes produced in fractions 3, 4 and 5, so the fractions were pooled into one single solution named fraction 3 (F3). Each solution group was further diluted into concentrations of 5 mg/ml, 10 mg/ml and 15 mg/ml with a 1% dimethylsulphoxide (DMSO) solution, which allowed for a constant emulsification factor through the assay tests, following similar methodologies implemented by Tayo *et al.* (2014) and Cabardo Jr and Portugaliza (2017). Positive controls of 0.5% albendazole and 0.5% ivermectin were prepared by dilution with distilled water (DW) and sonicated in an ultrasound bath for approximately 5 min to allow for optimal dilution (Tayo *et al.*, 2014; Cabardo Jr and Portugaliza, 2017). The negative control group solution was a 1% DMSO solution with distilled water (Ondua *et al.*, 2021).

3.9 Recovery and preparation of *Haemonchus contortus* eggs

The *H. contortus* eggs were recovered following the methods of Hubert and Kerboeuf (1992). All *H. contortus* eggs were collected from the rectum of 12 sheep that were infected by *H. contortus* as faecal samples at the University of Pretoria Innovation Africa, Hatfield, Pretoria. These sheep were chosen by the monthly recorded FAMACHA (Jurasek *et al.*, 2010) values taken at the University of Pretoria Future Africa farm. The samples weighed approximately 10-15 g and were deposited into zip-lock bags and transported in an ice-filled cooler to avoid any egg or larval development.

Methods described by Jurasek *et al.* (2010) were followed with modifications and discussed below. A fresh 2 g sample was deposited into a 250 mL beaker with water and mixed thoroughly to create a faecal slurry. This slurry was sieved to remove any large faecal particles from the solution. The solution was then mixed with a sugar water solution (1:25 ratio). The solution was left to stand for 15 mins allowing the eggs to float to the top. A small sample was taken and placed into a McMaster slide to allow for egg counting and establish a baseline egg number per gram (EPG) (Ondua *et al.*, 2021). After this baseline was established, the solution was sieved through a 25 µm sieve to remove any more unwanted faecal matter. Another 10 mL of sugar water was added to the sieved solution, thereafter the solution was left in a cool, dry place to allow the eggs to float to the surface. The top 5-10mL of the surface of the solution was deposited into a 25mL centrifuge tube and transported to the Phytomedicine Programme lab,

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Department of Paraclinical Sciences, Faculty of Veterinary Science, University of Pretoria, where the samples were stored at 5-10ºC.

3.10 Preparation of *Haemonchus contortus* larvae

The procedure followed the method of Hubert and Kerboeuf (1992) with some modifications. Faecal matter from the same samples obtained above were weighed (5-10 grams) and mixed with equal parts of water to form a homogenised slurry. Approximately 25% of the mixture was taken and mixed with vermiculite for the sole purpose of coproculture (Jurasek *et al.*, 2010) and larval identification. This solution mixed with vermiculite was deposited into a 1 litre jar with substrate to allow a culture to form. This jar was stored in a warm and humid environment to allow better development of the L3 *H. contortus*. After the desired development of L3 *H. contortus* larvae was reached, the sides/wall of the jar was rinsed with distilled water and deposited into a 25 mL centrifuge tube. This was then used for the larval assay discussed below.

3.11 Egg hatch assay

The egg hatch assay was based on a combination of methods used by Coles *et al.* (1992), Cabardo Jr and Portugaliza (2017) and Ondua *et al.* (2021). Every treatment was conducted in four replicates, with each replicate containing 50 µl of egg solution with an approximate number of 10-45 suspended eggs in a distilled water solution. Each replicate was placed in one well of a sterilized 96 well microtitre plate.

Firstly, 10 mL of the egg solution was transferred to a 25 mL centrifuge tube, where the eggs were concentrated to achieve an approximate amount of 10-45 eggs per 50 µL. This was done by placing the 10 mL egg solution in a refrigerated area at 5-10ºC to allow the eggs to float to the top of the solution in the container. The container was tilted between 15º - 20º and 50 µL was pipetted into wells of a flat bottomed 96-well microtitre plate. If the egg concentration was less than 10 eggs per 50 µL (or per well) then the solution was refrigerated again to allow the eggs to float to the top of the solution in the container, or another aliquot of the egg solution was transferred to another 25 mL centrifuge tube to form a more concentrated solution. Where numbers were greater than 45 eggs per 50 µL, further aliquots of 5mL of water were added to decrease the egg concentration until the correct egg concentration was achieved.

Following the procedure described by Cabardo Jr and Portugaliza (2017), the number of unhatched eggs in each well of the microtitre plate were counted and recorded. Thereafter each well was exposed to 50 µL of either the seed and leaf extracts, as well as the fractions of each, with control groups receiving 50 µL of 0.5% albendazole (positive control) and 1%

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DMSO/distilled water solution (negative control). These were incubated in a Labotec Incotherm incubator at 27ºC for 72 hrs. After the incubation period, the development of eggs was halted with addition of a drop of Lugol's iodine and the number of hatched free stage 1 larvae (L1) was counted and recorded (Cabardo Jr and Portugaliza, 2017). The mortality % of the eggs was calculated as follows:

$$\text{Mortality} = \frac{\text{Number of eggs before incubation} - \text{Number of free stage 1 Larvae (L1) after incubation}}{\text{Number of eggs before exposure to extracts or fractions}}$$

Figure 3.1 and 3.2 represents the egg hatch assay of *H. contortus* eggs after being exposed to *M. oleifera* extracts after 72 hours. (a) remained morulated, (b) showed larvae that failed to eclode and (c) free larval stage 1 (L1). These stages were illustrated in Cabardo Jr and Portugaliza (2017) and were used as reference for recording, with Figure 3.1 below illustrating the same egg illustrations found in this study (Figure 3.2).

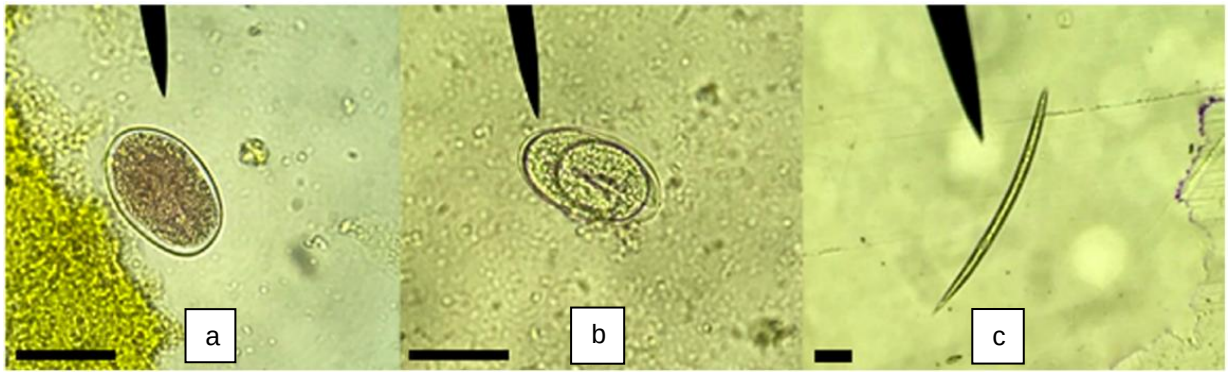

Figure 3. 1: Figures (a), (b) and (c) represents unmorulated eggs, egg failing larval eclosion and hatched larvae (Sourced from Cabardo Jr and Portugaliza, 2017)

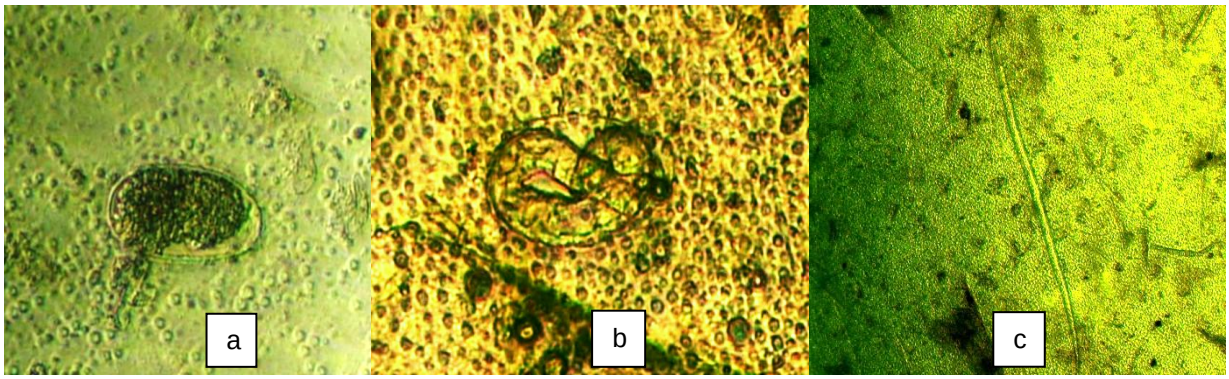

Figure 3. 2: Figures (a), (b) and (c) represent photographs of an unmorulated egg, egg failing larval eclosion and hatched larvae taken in this study.

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3.12 Larval survivability assay

The larval survivability assay was based on a combination of methods as discussed in Cabardo Jr and Portugaliza (2017), Tayo *et al.* (2014) and Coles *et al.* (1992) with modifications. Every treatment was tested in four replicates, with each replicate containing 50 µl of larval suspension with an approximate number of 10-25 randomly selected samples of larvae in a sugar water solution.

Firstly, 10 mL of the suspended larval solution was transferred to a 25 mL centrifuge tube, where the solution was concentrated to achieve an approximate amount of 10-25 larvae per 50 µL (Coles *et al.*, 1992; Tayo *et al.*, 2014). This was done by refrigerating the larval solution at 5-10ºC for 30 minutes to allow the larvae to sink to the bottom of the solution in the container. The solution at the bottom of the container was mixed by carefully aspirating and expelling 50 µL of the solution twice, with a third 50 µL aliquoted into the correctly marked well of a flat bottomed 96-well microtitre plate. Larvae in each well were counted using a Nikon Eclipse Ts2 Inverted microscope. If the larval number was less than 10 larvae per 50 µL, then the solution was refrigerated again to allow the larvae to sink and a further 5 mL from the original larval solution added to concentrate the numbers. This was done until the correct larval concentration was achieved. Similarly, if numbers were higher than 25 larvae per 50 µL, 5mL of water were added at a time until the correct larval concentration was achieved.

Following addition of the 50 µL larval solution to microtitre plate wells, the number of mobile larvae in each well of the flat bottomed 96-well microtitre were counted and recorded. Subsequently, each well was exposed to 50 µL of the seed and leaf extracts as well as the fractions of each, with control groups receiving 50 µL of 0.5% ivermectin (positive control) and 1% DMSO/distilled water solution (negative control) (Tayo *et al.*, 2014; Cabardo Jr and Portugaliza, 2017). Each well also received a drop of 1% Lily-Mayer's Haematoxylin, a stain which indicates if cuticle damage is present, and the plates were incubated at 27ºC for 24 hours (Coles *et al.*, 1992). After the incubation period, the number of live larvae were counted and recorded. Live larvae were identified through frantic and/or lazy movement after being exposed to light for 15 minutes to encourage the activity. The mortality % was calculated as follows:

$$\text{Mortality \%} = \frac{\text{Number of live stage 3 larvae before incubation} - \text{Number of live stage 3 larvae after incubation}}{\text{Number of live stage 3 larvae before incubation}}$$

Figure 3.3 expresses the before and after treatment effects on *H. contortus* L3 identified by Cabardo Jr and Portugaliza (2017), and was used as reference. (a) represents L3 that were motile but weak and (b) shows immobile L3 after prodding and/or stained pink with 1% Lily-

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Mayer's Haematoxylin stain. Figure 3.4 below shows photos taken as mentioned above, with (c) illustrating dead larvae and (d) illustrating live larvae.

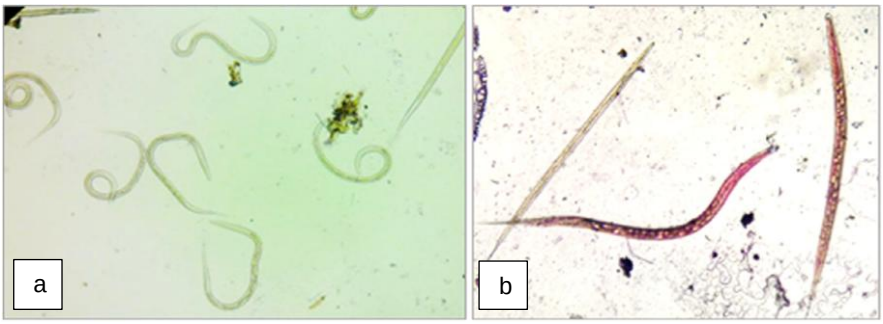

Figure 3. 3: Figures representing the before and after scenarios of larvae exposed to the crude extract (sourced from Cabardo Jr and Portugaliza, 2017)

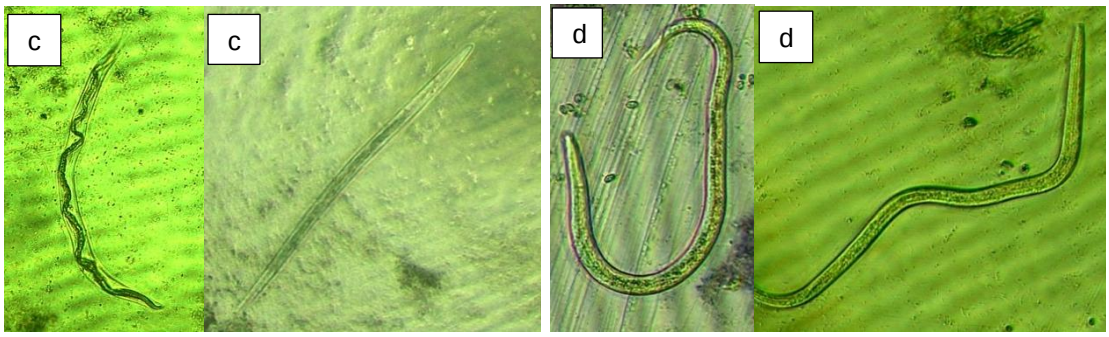

Figure 3. 4: Representation of dead and live larvae after exposure to extract done in this study. Figure (c) shows dead larvae and (d) represents live larvae.

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3.13 Statistical analysis

Data were analysed statistically with the Proc Mixed model (Statistical Analysis System, 2023) for the average effects. Means and standard error were calculated and significance of difference ($P<0.05$) between means was determined by Fischer's test (Cornell, 1989).

1. Model for within crude extract.

The linear mix model used is described by the following equation:

$$Y_{ijk} = \mu + P_i + E_j + C_k + PE_{ij} + PC_{ik} + EC_{jk} + PEC_{ijk} + e_{ijk}$$

Where Y_{ijk} = variable studied during the period

μ = overall mean of the population

P_i = effect of the i^{th} plant material

E_j = effect of the j^{th} egg hatch assay (EHA), larval survivability assay (LSA)

C_k = effect of the k^{th} concentration

PEij = effect of the ij^{th} interaction between plant material and EHA/LSA

PCik = effect of the ik^{th} interaction between plant material and concentration

ECjk = effect of the jk^{th} interaction between EHA/LSA and concentration

PECijk = effect of the ijk^{th} interaction between plant material, EHA/LSA and concentration

e_{ijk} = error associated with each Y

2. Model within each fraction

$$Y_{ij} = \mu + PC_{ij} + e_{ijk}$$

Where Y_{ij} = variable studied during the period

μ = overall mean of the population

PC_{ij} = effect of the ij^{th} class and concentration

e_{ij} = error associated with each Y

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Chapter 4: Results & Discussion

4.1 Nutrient composition of *Moringa oleifera* samples

Moringa oleifera has drawn significant attention in scientific literature due to the high nutrient density it possesses and the potential application this plant can have in addressing nutritional deficiencies. A proximate analysis was done on the Moringa oleifera samples used in this study to validate its nutrient composition and further emphasise its potential use in this research field. Table 4.1 illustrates the values obtained from the proximate analysis conducted on *M. oleifera* leaves and seeds obtained in this study and compared to *M. oleifera* proximate analysis reported in a study by Su and Chen (2020). The values for *M. oleifera* obtained in this study are similar to those in literature, with minor differences in crude protein, with levels of 33.7% and 28.3% for *M. oleifera* leaves and seeds (respectively) used in this study versus 23.0-30.3% level found in literature (Su and Chen, 2020). *M. oleifera* leaves contained higher levels of crude protein and ash in relation to *M. oleifera* seeds (33.7% versus 28.3% and 8.6% vs 3.8%). The seed fraction of *M. oleifera* had higher levels of crude lipid and fibre (25.7% and 22.1%) than the leaf fraction (3.77% and 8.1%), which could be due to seed hulls being present in the meal. The differences between the *M. oleifera* materials used in the study and those used in previous studies may be due to environmental differences as well as different cultivar locations, meaning external factors like these mentioned above could cause variation in nutritional characteristics of *M. oleifera* (Moyo *et al.,* 2011).

Table 4.1: Nutritional value of *Moringa oleifera* versus other plant species used in livestock production (% dry matter basis) .

Feedstuff category	Plant species	Crude Protein	Crude lipid	Crude fibre	Ash
Woody plant forages	*M. oleifera*	23.0-30.3	7.09	5.9	7.6-12
	M. oleifera/ leaves[A]	33.7	3.77	8.1	8.6
	M. oleifera/seeds[A]	28.3	25.7	22.1	3.8
	Morus alba	21.2-29.8	5.5	6.9	11.6
	Broussonetia papyrifera	21.0	3.2	9.1	12.1
	Caragana korshinskii	9.9	3.2	34.4	6.7
Conventional crop forages	Alfalfa meal	19.1	2.3	22.7	7.6
	Soybean meal	25.5	17.3	4.3	4.2
	Maize meal	9.4	3.1	1.2	1.2

Adapted and sourced from Su and Chen, 2020
Superscript A: *M. oleifera* leaf and seed material obtained in this study

4.2 Anthelmintic potential of *Moringa oleifera* leaf and seed crude extracts

Table 4.2 illustrates the mean mortality values of *H. contortus* eggs and larvae (L3) due to the anthelmintic activity of the crude extract of *M. oleifera* leaves and seeds in the egg hatch assay (EHA) and larval survivability assay (LSA). The highest average ovicidal and larvicidal activity, 88.6% and 45.4% respectively, resulted from exposure to the *M. oleifera* leaf extracts. The larvicidal assays were generally dose dependent, exhibiting higher mortality percentages with

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increasing concentration of the test substances, whereas the ovicidal assays showed a non-dose dependent trend as the concentrations increased, which could be due to different secondary metabolites influencing each other (Acamovic and Brooker, 2005; Arroyo-Lopez *et al.*, 2014; Klongsiriwet *et al.*, 2015; Ondua *et al.*, 2021).

The only significant difference ($P < 0.05$) in the EHA was observed with the *M. oleifera* extract between 10 mg/ml and 15 mg/ml concentrations, where larvae exposed to a concentration of 15 mg/ml had the highest mortalities (100,0%). Significant differences were also observed with the *M. oleifera* seed extract between 5 mg/ml and 10 mg/ml ($P<0.05$), as well as between 10 mg/ml and 15 mg/ml ($P<0.05$). In the EHA, the highest mean mortality percentage was observed with the *M. oleifera* seed and leaf extracts (84.0% and 88.6% respectively). The lowest mortality percentages observed in the LSA were 26.6% in the seed crude extract and 45.4% in the leaf crude extract. It should also be noted that most of the egg mortalities were considered to be eggs that failed larval eclosion, where a developed larva was observed inside the egg but which failed to hatch (Cabardo Jr and Portugaliza, 2017).

The highest ovicidal mortality percentage was observed in the EHA with the *M. oleifera* leaf extract with 100% efficacy at 15 mg/ml, followed with 95% mortality efficacy at 10 mg/ml of *M. oleifera* seed extract. The EHA of *M. oleifera* seeds showed a lower reaction in the 15 mg/ml CE concentration (76,5%), whereas a stronger anthelmintic response was observed in the 5 and 10 mg/ml crude extract concentrations (80,7% and 95,0%, respectively). In contrast, the 5 -and 10 mg/ml crude extract concentrations (89,0% and 76,7%, respectively) were lower than that of the 15 mg/ml in the ovicidal assay in leaves, with the 10 mg/ml concentration showing the lowest anthelmintic activity and 15 mg/ml expressing the highest at 100%, higher than that of the positive control at 98,3% ($P<0.05$).

In the assays done with the *M. oleifera* crude extracts and fractions thereof, it was found that the majority of the unhatched *H. contortus* eggs had formed larvae inside the unhatched cell. Although not as prevalent as the bulk of the unhatched eggs reported in this study, this was also discovered in a study conducted by Cabardo Jr and Portugaliza (2017). In contrast, Vargas-Magañ̃a *et al.* (2014) found similar hatching results through conducting a study with different acetone-water extracts to investigate potential anthelmintic properties towards the hatching assays of *H. contortus* where the eggs failed larval eclosion, meaning the unhatched eggs exhibited developed larvae. They speculated that some of the secondary phytochemical compounds might have different mechanisms that can influence eclosion of the eggs. These mechanisms could be due to the bioactive compounds found in the extracts that can influence the permeability of the cell walls of the eggs by influencing the lipoproteins of the membranes

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which inhibits the normal release of certain compounds and negatively affecting the osmotic pressure in the egg, causing impaired hatching where the egg failed to eclose (Vargas-Magañ*a et al.*, 2014). Another mechanism that might be a potential effect and mentioned in the study done by Vargas-Magañ*a et al.* (2014) was the process of inhibiting the enzymes responsible for the hatching process, where the phytochemical compounds bind to the enzymes, obstructing the action of cell wall degrading substances (Molan *et al.*, 2002). Vargas-Magañ*a et al.* (2014) discussed a possible third mechanism, where there might have been contesting hatching factors on the cell membrane of the eggs, where the active phytochemical compounds might be binding to these factors that disrupt the hatching process (Craig *et al.*, 2007; Doncaster and Sheperd, 1967).

These proposed hatching inhibition mechanisms could be the reason for the dose dependency as reported in above mentioned studies by Vargas-Magañ*a et al.* (2014) and Cabardo Jr and Portugaliza (2017). Salles *et al.* (2014) reported the same failed eclosion results with an approximate 90% egg inhibition with *M. oleifera* seed extracts. The study further investigated the mode of inhibition by running a membrane analysis assay, where it was found that the bioactive compounds with a kilodalton (kDa) of below 12 were responsible for the high egg hatch inhibition rather than compounds with a kDa above 12. The study also deduced that tannins, terpenes and alkaloids were also detected in these fractions as the primary bioactive compounds. These compounds were also found in the *M. oleifera* crude extract in the present study, analysed by UPLC-UV-QTOF/MS, and may be responsible for the high numbers of failed eclosion eggs found in the assays (Hernandez-Villegas *et al.*, 2011; Cabardo Jr and Portugaliza, 2017).

In the larvicidal assay where the concentrated *M. oleifera* leaf solutions were used, the 5 mg/ml concentration had the lowest significant ($P<0.05$) mortality rate (30,1%) versus the 10 mg/ml and 15 mg/ml concentrations, at 51,8% and 54,3% respectively. Tayo *et al.* (2014) reported dose dependent interactions, suggesting that as the concentration of the solutions increases, and there for the bioactive compound concentrations as well, so do the mortality of *H.contortus*. Tayo *et al.* (2014) regarded the ethanolic extracts as the most effective against the free living stages of *H. contortus*, with 98.8% ± 2.5% and 100% ± 0% mortality rate for stage 1(L_1) and 2(L_2) larvae respectively, at a concentration of 5 mg/ml of *M. oleifera* leaf ethanolic extracts. These values are higher than what was found in the present study, at 30.1% ± 5.72%. This could be due to the fact that the life stage of the *H. contortus* larvae used in this study was stage 3 (L_3) larvae and can be possibly explained by understanding that the L_3 are less vulnerable to the bioactive compounds present in the solutions versus the L_2 and L_1 because these larvae still in their vulnerable developmental stages (Ponè *et al.*, 2010).

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In the *M. oleifera* leaf EHA, the 5 mg/ml -and 10 mg/ml concentration solution group (CSG) were insignificantly (P<0.05) lower (89,0% and 76,7%, respectively) than the positive control group (98,3%), with the 15 mg/ml CSG being significantly (P<0.05) higher (100,0% ± 8.09%). In contrast, the *M. oleifera* seed EHA followed the same dose dependency, but where the 10 mg/ml CSG had the highest mortality level, at 95,0% ± 8,09%, nearly equal to that of the positive control group (P>0.05), at 97,9%. This dose dependency could be due to certain bioactive compounds having a synergistic effect on the *H. contortus* eggs in the *M. oleifera* leaf EHA and an additive effect in the *M. oleifera* seed EHA (Hernandez-Villegas *et al.*, 2011).

In both the larvicidal assays containing the *M. oleifera* crude leaf and seed extracts, the positive control mortality levels were significantly (P<0.05) higher, at 94,1% and 84,6%. These lower levels could be due to the L3 larvae being less vulnerable to the bioactive compounds present in the *M. oleifera* crude leaf and seed extracts (Ponè *et al.*, 2010).

Across *M. oleifera* leaves and seeds were multiple significant differences (P < 0.05) between the different assays, but more so for crude extract concentration solution groups of 10 mg/ml (CSG-10) and 15 mg/ml (CSG-15) than concentration solution group 5 mg/ml (CSG-5). The CSG-5 *M. oleifera* leaf assays was overall higher (89,0% for EHA and 30,1% for LSA) than the CSG-5 mg/ml seed assays (80,7% for EHA and 25,6% for LSA). The same was observed for the 15 mg/ml CSG, where the assays, EHA and LSA, for *M. oleifera* leaves were 100,0% and 54,3% and for *M. oleifera* seeds were 76,5% and 28,9%. The CSG 10mg/ml was the only solution group to differ, where the EHA in the *M. oleifera* seed crude extract solution showed a higher mortality level than in the *M. oleifera* leaf crude extract solution (95,0% versus 76,7%). This could be due to certain bioactive compounds being more prevalent in certain parts of the plant than others (Salles *et al.*, 2014).

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Table 4.2: Different mean mortality percentages regarding the *in vitro* anthelmintic activity of *Moringa oleifera* leaf and seeds crude extract (CE) over the egg hatching assay (EHA) and larval survivability assay (LSA).

	Leaves				Seeds			
Concentration	**EHA**	**LSA**	$\bar{x}$	**±SE**	**EHA**	**LSA**	$\bar{x}$	**±SE**
5	$89{,}0^{a}{}_{xy}$	$30{,}1^{b}{}_{y}$	59,5	8,09	$80{,}7^{a}{}_{y}$	$25{,}6^{b}{}_{y}$	53,2	8,09
10	$76{,}7^{a}{}_{x}$	$51{,}8^{b}{}_{x}$	64,2	8,09	$95{,}0^{c}{}_{x}$	$25{,}1^{d}{}_{y}$	60,1	8,09
15	$100{,}0^{a}{}_{y}$	$54{,}3^{b}{}_{x}$	$77{,}1^{A}$	8,09	$76{,}5^{c}{}_{y}$	$28{,}9^{d}{}_{y}$	$52{,}7^{B}$	8,09
Positive Control	$98{,}3_{x}$	$94{,}1_{w}$	96,2		$97{,}9_{x}$	$84{,}6_{x}$	91,2	
Negative control	$11{,}6_{z}$	$12{,}4_{z}$	12.0^{A}		$7{,}0_{z}$	$3{,}5_{z}$	$5{,}2^{B}$	
±SE	5,72	5,72	4,04		5,72	5,72	4,04	
$\bar{x}$	$88{,}6^{A}$	$45{,}4^{B}$	-		$84{,}0^{A}$	$26{,}6^{C}$	-	

abc… = Row means with different superscript letters differ with a P < 0.05.
wxyz = Column means with different subscript letters differ with a P < 0.05.
ABC = Row means with different superscript differ P < 0.05.
EHA: Egg Hatch Assay, LSA: Larval Survivability Assay

UPLC-UV/QTOF/MS analysis was used to characterise the different bioactive compounds found in the experimental *M. oleifera* leaf and seed plant material. Table 4.3 below lists the different bioactive compounds found in the *M. oleifera* crude leaf and seed extracts used in the present study, as well as the metabolite classes these compounds belong to. Terpenoids, flavonoids, alkaloids, and cinnamic acids have all been shown in numerous studies to have anthelmintic activity against a variety of gastrointestinal nematode (GIN) species and are cross referenced in Table 4.3 to the specific bioactive compound found in the *M. oleifera* plant material in the present study. The chemical classes mentioned in Table 4.3 below correspond with the literature findings, which may account for the anthelmintic efficacy of *M. oleifera* leaves and seeds utilised in this investigation against *H. contortus*.

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Table 4. 3: Different bioactive compounds with their respective phytochemical classes from leaf and seed crude extracts of *Moringa oleifera, as well as* previous studies showing activity against GIN

Plant Material	Compound name	Compound classes	References
Leaf^{+}	Dehydronuciferine	Aporphine alkaloids	Mumed *et al.* (2022), Ali *et al.* (2021), Salles *et al.* (2014)
	1-Ethyl-4-methoxy-β-carboline	Carboline alkaloids	
	Picrasidine-J		
	Harmaline		
	2-Phenazinecarbonitrile	Phenazine alkaloids	
	4(3H)-Quinazolinone	Quinazoline alkaloids	
	Allantoin	Purine alkaloids	
	Hirsutine	Tryptophan alkaloids	
	N-(4-Chlorobutyl) butanamide	Polyamines (Ornithine alkaloids)	
	Quercimeritrin	Flavonols (Flavonoids)	Mumed *et al.* (2022), Ali *et al.* (2021), Silva *et al.* (2019)
	4,7-Dimethoxyl-5-coumarin	Simple coumarins	Mumed *et al.* (2022), Silva *et al.* (2019)
	5,7,8,2′-Tetrahydroxy-flavone-7-O-β-D-glucoside	/	
	6,7-Dihydroxy-2-(2-phenylethyl) chromone	Chromones	
	6-Methoxy-2-[2-(3'-methoxyphenyl) ethyl] chromone		
	Deoxypaeonisuffrone	Pinane monoterpenoids	Mumed *et al.* (2022), Salles *et al.* (2014)
	Pseudolaric acid B	Diterpenoids	
	Deacetylmatricarin	Guaiane sesquiterpenoids	
	Bistortaside	Tannin	Mumed *et al.* (2022), Salles *et al.* (2014), Vargas- Magaña *et al.* (2014)
	Aloenin	Cyclic polyketides	
	Leonurine	Simple phenolic acids	Mumed *et al.* (2022)
	Lignoceryl ferulate	/	
	Methyl ophiopogonanone B	Shikimates and Phenylpropanoids	Herath *et al.* (2019)
	p-Hydroxyphenol (5-Methyl-2-furaldehyde)	/	
Leaf $^{-}$	3,5,7,2′, 6′-Hydroxy-flavone 5-2′-O-β-D-glucopyranoside	Flavonoids	Mumed *et al.* (2022), Ali *et al.* (2021), Silva *et al.* (2019),
	3,8-Di-C-glucosylapigenin		
	6-Hydroxykaempferol-3-O-glucoside	Flavonols (SC: Flavonoids)	Mumed *et al.* (2022), Ali *et al.* (2021), Silva *et al.* (2019)
	Kaempferol-3-O-neohesperidoside		
	Kaempferol-3-O-rutinoside		
	Quercetin-3-O-rutinoside_1 (Rutin)		
	Kushenol - X	Dihydroflavonols (SC: Flavonoids)	
	(+)-Praeruptorin E	Pyranocoumarins	

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Table 4. 3: Different bioactive compounds with their respective phytochemical classes from leaf and seed crude extracts of *Moringa oleifera,* as well as referenced studies showing activity against GIN

Plant Material	Compound name	Compound classes	References
Leaf $^{-}$	Dehydronuciferine	Aporphine alkaloids	Mumed *et al.* (2022), Ali *et al.* (2021), Salles *et al.* (2014)
	Anhydroberberillic acid	SC: Tyrosine alkaloids	
	Kaempferol-3-O-(6″-O-acetyl)-β-D-glucopyranoside	Flavonoid glycosides	
	Biflorin	Chromones	
	Cimicifugic acid A	Cinnamic acids and derivatives	Mancilla-Montelongo *et al.* (2019), Katiki *et al.* (2017), Camurca-Vasconcelos *et al.* (2007), Pessoa *et al.* (2002)
	Aspon	Pathway: Fatty acids	Pineda-Alegría *et al.* (2020)
	Luteolin 7-O-β-D-(6''-acetyl)-glucopyranoside	/	
Seed^{+}	13,17-Epoxy alisol A	Terpernoids	Mumed *et al.* (2022), André *et al.* (2020), Katiki *et al.* (2019), Katiki *et al.* (2017), Qi *et al.* (2015), Zhu *et al.* (2013), Foster *et al.* (2011), Shai *et al.* (2009)
	1-Vinyl-4-methoxy-β-carboline	/	
	N,N,N-Trimethyltryptophan	Simple indole alkaloids (Tryptophan alkaloids)	Mumed *et al.* (2022), Salles *et al.* (2014)
Seed^{-}	4',5,6,7-Tetramethoxy-flavone	Flavones (SC: Flavonoids)	Mumed *et al.* (2022), Silva *et al.* (2019)
	5-Hydroxyauranetin	Flavonols (SC: Flavonoids)	
	7-Ketoologanin	/	
	Aspon	Pathway: Fatty acids	Pineda-Alegría *et al.* (2020)
	Cniforin B	Simple coumarins/ Furocoumarins	
	Ethylnotopterol	/	
	Sweroside	Secoiridoid monoterpenoids	André *et al.* (2020), Katiki *et al.* (2019), Katiki *et al.* (2017), Zhu *et al.* (2013).
	Syringin	Menthane monoterpenoids	

(+) superscript shows positive ion binding in UPLSC test.
(-) superscript shows negative ion binding in UPLSC test.

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4.3 The anthelmintic effect of *Moringa oleifera* leaf crude extracts and fractions on *Haemonchus contortus* eggs and larvae

4.3.1 The egg hatch assay with *Moringa oleifera* leaf crude extract and fractions

The egg hatch assay (EHA) conducted with *M. oleifera* crude extracts as well as the fractions prepared from the crude extracts showed the highest mortality levels of all the assays done in this study, with the average mortality of the crude extract (CE) as well as fractions 2 (F2), 3 (F3), 4 (F4), 5 (F5), 6 (F6), and 7 (F7) being comparable (P>0.05) to the mortality level of the positive control group (PCG), as seen in Figure 4.1 below. Mumed *et al.* (2022) reported similar results, where the anthelmintic activity of crude extracts of *Nicotiana tabacum, Zingiber officinale and Croton macrostachyus* were examined, with mortality results (93% for *C. macrostachyus*, 83% for *N. tabacum*, and 50% for *Z. officinale*) comparable to that of the positive control (P>0.05).

Fraction 6 had the highest mean mortality rate at 96.9% of all the fractions and fraction 1 (F1) having the lowest mean mortality level at 88.6%. Within the fraction 6 treatment, the mortality levels were comparable (P>0.05) between the different concentration groups (5, 10, and 15 mg/ml) with 5 mg/ml and 10 mg/ml having the highest at 100.0% and 15 mg/ml having the lowest at 90.6%. Both F6 and F3 had a negative dose response, meaning the mortality rates decreased (100.0% to 90.6% for F6 and 97.5% to 83.4% for F3) as the concentration increased (5mg/ml – 15mg/ml). The negative dose response witnessed in the present assay could be due to an antagonistic effect caused by an increase in concentration of a specific molecule that could result in decreasing mortality as the concentration of the solution increases (Vargas-Magaña *et al.*, 2014).

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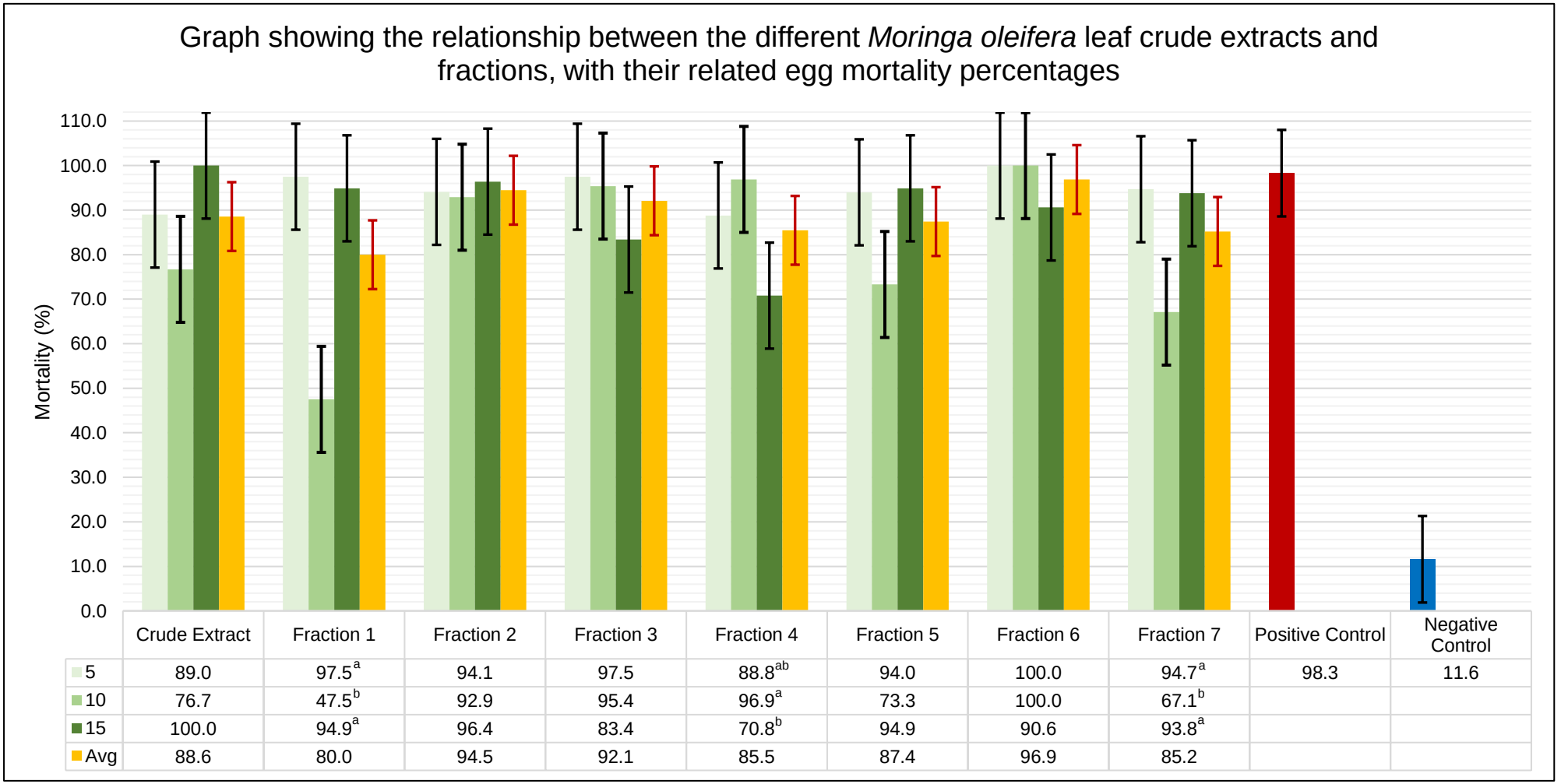

	Crude Extract	Fraction 1	Fraction 2	Fraction 3	Fraction 4	Fraction 5	Fraction 6	Fraction 7	Positive Control	Negative Control
5	89.0	97.5^{a}	94.1	97.5	88.8^{ab}	94.0	100.0	94.7^{a}	98.3	11.6
10	76.7	47.5^{b}	92.9	95.4	96.9^{a}	73.3	100.0	67.1^{b}		
15	100.0	94.9^{a}	96.4	83.4	70.8^{b}	94.9	90.6	93.8^{a}		
Avg	88.6	80.0	94.5	92.1	85.5	87.4	96.9	85.2		

Figure 4. 1: Graph showing the relationship between different *Moringa oleifera* leaf extracts and fractions and their related egg mortality percentages

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4.3.2 The larval survivability assay with *Moringa oleifera* leaf crude extract and fractions

The larval survivability assay (LSA) conducted with *M. oleifera* leaf crude extracts as well as the fractions of the crude extracts with the mortality levels of each are presented in Figure 4.2 below. The crude extract (CE) had the lowest mean mortality level at 45.4%. Similar to the EHA above, F1 had the lowest mean mortality of 58.8% as well as F6 having the highest mortality mean at 94.5% followed by F7 at 86.1%.

Fraction 1, 2, 5, and 7 had a positive dose response, meaning as the concentration of the fraction increased (5 mg/ml – 15 mg/ml), the mortality levels increased as well (Figure 4.2). Mphahlele *et al.* (2016) reported similar results where the increased concentrations of aqueous extracts of *Pygeum africanum* and *Setaria italica* (2.5 mg/ml, 5.0 mg/ml and 7.5 mg/ml) resulted in increased mortality rates for *P. africanum* (50.0%, 58.0% and 60%) and *S. italica* (59.0%, 64.33% and 70.0% respectively) crude extracts. In contrast, fraction 4 had a negative dose response, meaning as the fraction concentration increased, the mortality level decreased, which could be due to an antagonistic effect between the bioactive molecules (Vargas-Magañ*a et al.*, 2014). Fractions 2 and 5 had significant mortality rates for the concentration of 15 mg/ml ($P<0.05$) at 84.5% and 74.5%, which could mean that specific bioactive molecules were dose dependent as mentioned above (Vargas-Magañ*a et al.*, 2014).

The mean mortality rates of fraction 6 and 7 (94.5% and 86.1%) were both comparable ($P>0.05$) to the mortality levels of the positive control, which further shows the anthelmintic potential of *M. oleifera* (Cabardo Jr and Portugaliza, 2017)

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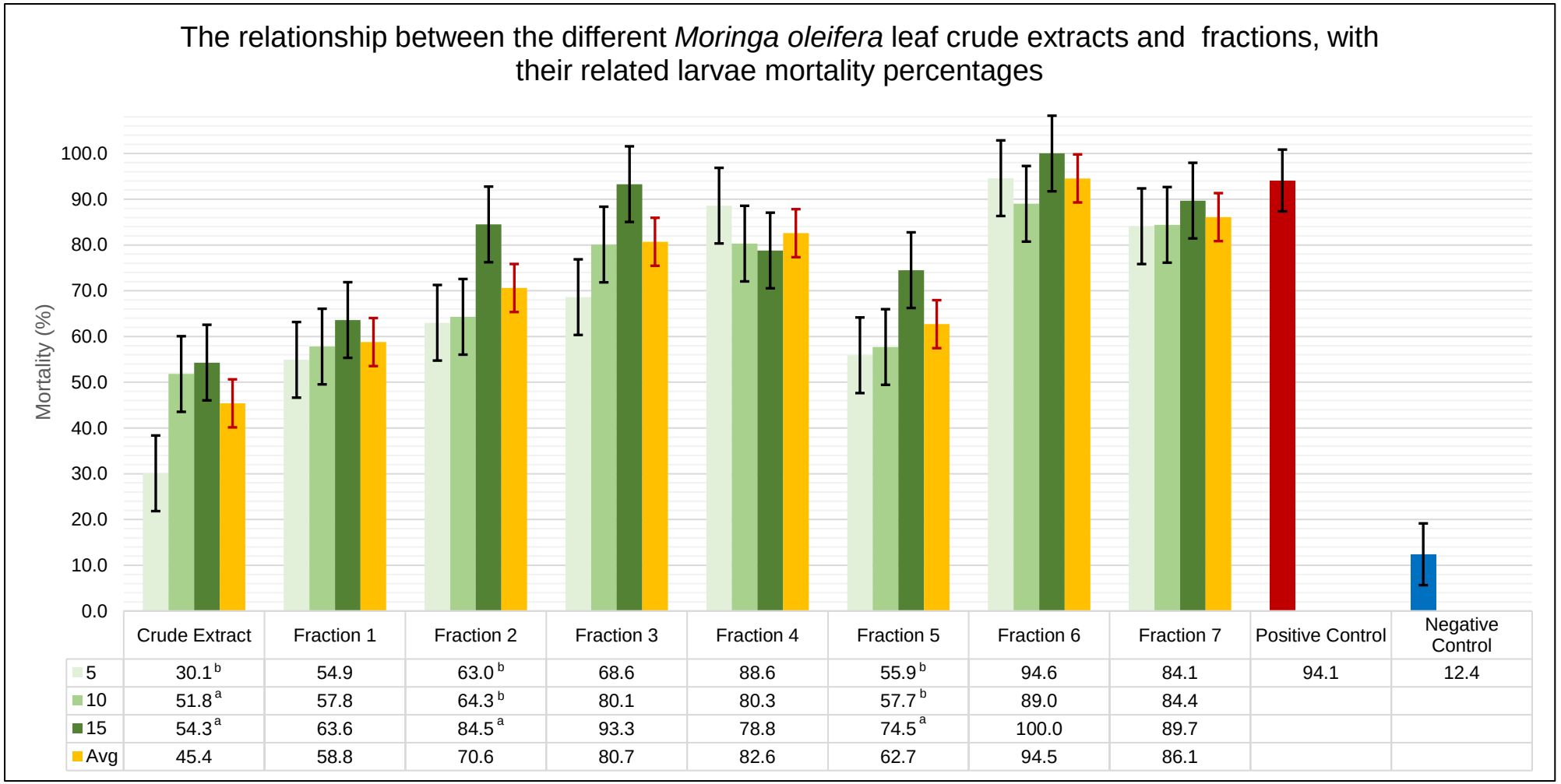

	Crude Extract	Fraction 1	Fraction 2	Fraction 3	Fraction 4	Fraction 5	Fraction 6	Fraction 7	Positive Control	Negative Control
5	30.1 [b]	54.9	63.0 [b]	68.6	88.6	55.9 [b]	94.6	84.1	94.1	12.4
10	51.8 [a]	57.8	64.3 [b]	80.1	80.3	57.7 [b]	89.0	84.4		
15	54.3 [a]	63.6	84.5 [a]	93.3	78.8	74.5 [a]	100.0	89.7		
Avg	45.4	58.8	70.6	80.7	82.6	62.7	94.5	86.1		

Figure 4. 2: The relationship between the different *Moringa oleifera* leaf extracts and fractions and their related larvae mortality percentages

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4.3.3 Fractionation of *Moringa oleifera* leaf crude extract and the anthelmintic effect against *Haemonchus contortus* eggs and larvae

Fraction 6 (F6) resulted in the highest average mortality percentages of the *M. oleifera* leaf crude extract assay used in the EHA and LSA, and was chosen to undergo UPLC-UV/QTOF/MS analysis to identify potentially relevant secondary metabolites that are smaller than 12 kilodaltons (KDa). Fraction 6 contained a large quantity of terpenoid compounds, more specifically sesquiterpenoids. These are represented by peaks A^- and C^- in figure 4.3 below. These secondary metabolites have a 15-carbon structure made up of three isoprene units called sesquiterpenoids, which may comprise germacranolides, eudesmanolides, guaianolides, and pseudoguaianolides, each distinguished by specific ring arrangements. Cinic acid, which was isolated by Arango-De-la-Pava *et al.* (2024) from *Artemisia* species, resembles a type of pseudoguaianolide sesquiterpene lactone, which exhibits significant biological potency due to its distinct structure, featuring an exocyclic methylene linked to a γ-carbonyl group. The biological activity of sesquiterpene lactones is attributed to the α-methylene, γ-lactone system, facilitating interaction with protein thiol groups, which may elucidate cinic acid's effectiveness against L3 infective larvae of *H. contortus* (Peña-Espinoza *et al.*, 2018). Oliveira *et al.* (2014) also reported that plant extracts with medium to high levels of sesquiterpenoids and monoterpenoids, showed the highest mean instance of eggs with failed eclosion (embryonated eggs). Thus, with these compounds having certain specific actions against protein thiol groups of *H. contortus* egg and larvae cell walls can be attributed to one or more of the mechanisms proposed by Vargas-Magañ a *et al.* (2014) mentioned above.

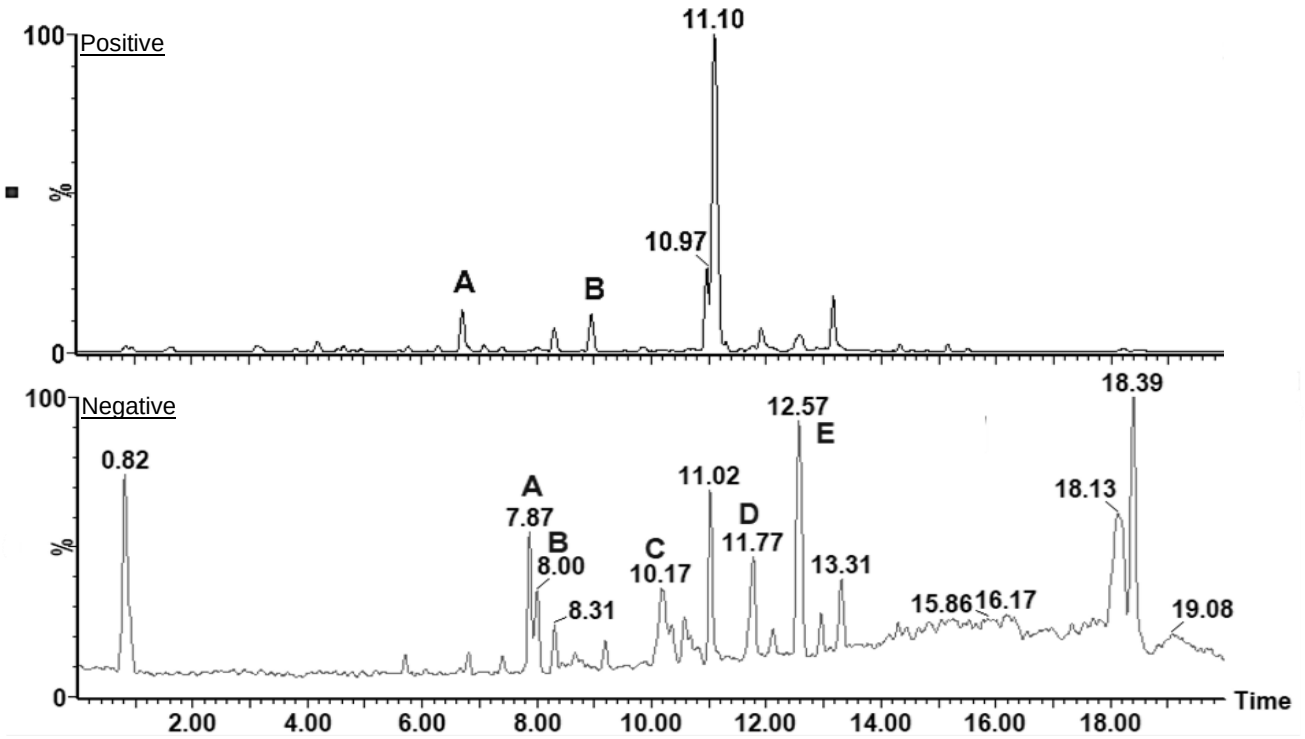

Figure 4. 3: *Moringa oleifera* leaf fraction 6 UPLC-UV-QTOF/MS positive and negative chromatograms

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Table 4.4: *Moringa oleifera* crude leaf extract with fraction 6 phytochemical compounds after UPLC-UV-QTOF/MS test

	Compound Name	Time	Compound Class
A^+	Digiprolactone	6.70	Apocarotenoids (β-)
B^+	3-Tert-butyl-4-methoxyphenol	8.95	Apocarotenoids (β-)
A^-	4,4'-bis[(1e)-2-[(1s,4as,8as)-5,5,8a-trimethyl-2-methylidene-hexahydro-1h-naphthalen-1-yl]ethenyl]-5h,5'h-[3,3'-bifuran]-2,2'-dione	7.87	Sesquiterpenoids
B^-	2-[(3s,4s,5r)-3-(hydroxymethyl)-5-(prop-1-en-2-yl)-1,2-dioxolan-4-yl]propan-2-yl acetate	8.02	Menthane monoterpenoid
C^-	Rubescensin O	10.16	Sesquiterpenoids
D^-	Aceroside XII	11.77	Linear diarylheptanoids
E^-	-	12.57	Fatty acid

Fraction 3 (F3) showed a distinct dosage response in the LSA and EHA, and was selected for further analysed using UPLC – UV/QTOF/MS and the observed compounds from the assay are shown in Table 4.5. Regarding the EHA, the increase in concentration was accompanied by a corresponding drop in mortalities, while in contrast to the LSA, an increase in concentration was accompanied by an increase in mortalities. The compound composition of F3 was found to comprise cinnamic acids and derivatives, identified in Table 4.5. Cinnamic acids could be responsible for the anthelmintic activity, as Mancilla-Montelongo *et al.* (2019) reported that isolated cinnamic acid and its derivatives, ferulic and chlorogenic acids, demonstrated significant anthelmintic hatching inhibition, with an effective concentration to kill 50% of the tested population (EC_{50}) at 1628.10 µg/mL and, in comparison, higher mortality in eggs that failed eclosion, as in the present study. These compounds, combined with the terpenoids (menthane monoterpenoid) found in F3, could be responsible for the different dose dependency as reported in Figure 4.2.

The negative dose dependency trend in the EHA of *M. oleifera* leaf F3 could be explained by different compounds acting synergistically or antagonistically with each other as the concentration increased. Páez-León *et al.* (2022) reported similar results where the EHA had a higher anthelmintic effect from *M. oleifera* leaves, contrary to the LSA. Páez-León *et al.* (2022) also found apocarotenoids (6-hydroxy-4,4, 7a-trimethyl-5,6,7, 7a-tetrahydrobenzofuran-2) in the extract through gas chromatography-mass spectrometry, which were also detected in F3 via UPLC – UV/QTOF/MS.

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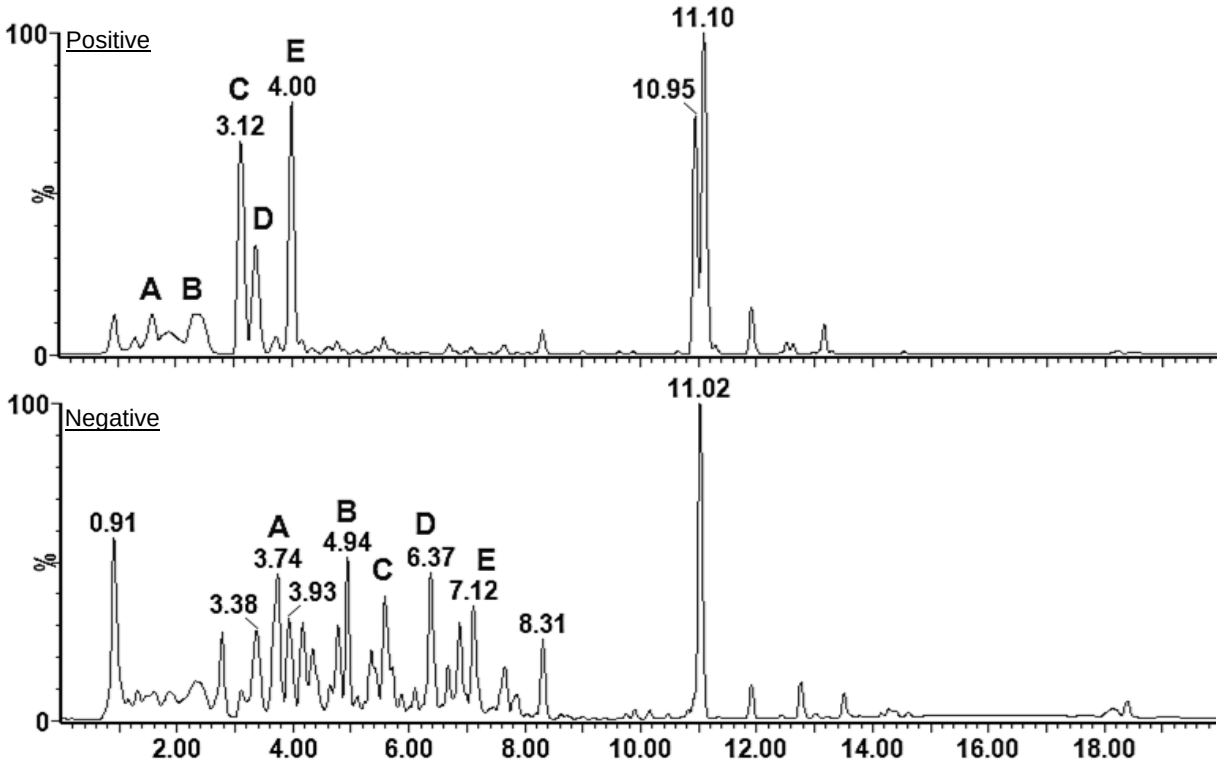

Figure 4. 4: *Moringa oleifera* leaf fraction 3 UPLC-UV-QTOF/MS positive and negative chromatograms

Table 4.5: *Moringa oleifera* crude leaf extract with fraction 3 phytochemical compounds after UPLC-UV-QTOF/MS test

	Compound Name	Time	Compound Class
A+	Methyl 3,4,5-trimethoxycinnamate	1.60	Cinnamic acids and derivatives
B+	Fructosyl isoleucine	2.34	Dipeptides
C+	Phenylpropionic acid	3.12	Shikimates and Phenylpropanoids
D+	N-({4-[(3,4,5-trihydroxy-6-methyloxan-2-yl)oxy]phenyl}methyl)methoxycarboximidic acid	3.37	-
E+	L-tryptophan	4.00	Amino acids and Peptides
A-	Chlorogenic acid	3.74	Cinnamic acids and derivatives
B-	Glucosinalbin 4-(4-acetylrhamnoside)	4.94	Glucosinolates
C-	3,8-Di-C-glucosylapigenin	5.59	-
D-	1,2,10-Trihydroxydihydro-trans-linalyloxide-7-O-beta-D-glucopyranoside	6.37	Acyclic monoterpenoids
E-	Nonioside D	7.12	Fatty acyl glycosides

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4.4 The anthelmintic effect of *Moringa oleifera* seed crude extracts and fractions

4.4.1 The egg hatch assay with *Moringa oleifera* seed crude extract and fractions

The results of the *H. contortus* egg hatch assay (EHA) conducted with the *M. oleifera* seed crude extract and fractions with the mortality levels of each are presented in Figure 4.5. Fraction 5 (F5) had the highest mean mortality rate, at 94.6%, with a positive dose response. The concentrations within F5 (5, 10, and 15 mg/ml) were comparable (P>0.05) to that of the positive control (97.9%), with mortality rates at 91.4%, 95.0% and 97.2%, respectively. The same results were reported by Cabardo Jr and Portugaliza (2017) where the statistical analyses showed no difference (P>0.05) between the *M. oleifera* crude extract and that of the positive control group (albendazole).

In addition, fractions 1 and 2 had positive dose responses as well, with results of the concentration groups 10 mg/ml (87.7% and 86.0%) and 15 mg/ml (90.8% and 94.8%) being comparable (P>0.05) to the positive control group. In fraction 2, the concentration treatments of 10 mg/ml and 15 mg/ml were significantly higher (P<0.05) than that of the 5 mg/ml concentration group (60.1%). In contrast, fraction 4 had a negative dose response, with the 5 mg/ml concentration group comparable (P>0.05) to that of the positive control group.

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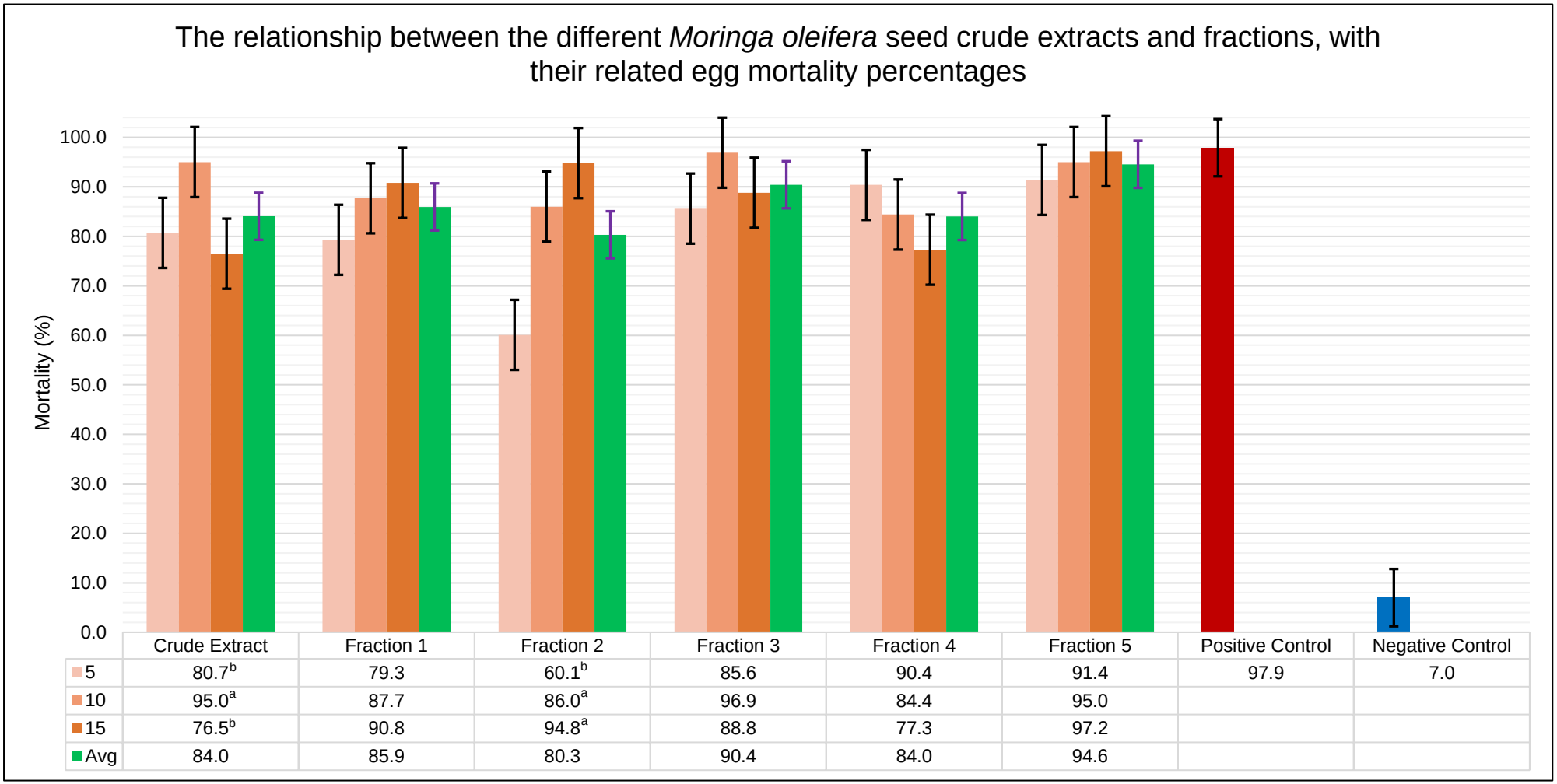

	Crude Extract	Fraction 1	Fraction 2	Fraction 3	Fraction 4	Fraction 5	Positive Control	Negative Control
5	80.7[b]	79.3	60.1[b]	85.6	90.4	91.4	97.9	7.0
10	95.0[a]	87.7	86.0[a]	96.9	84.4	95.0		
15	76.5[b]	90.8	94.8[a]	88.8	77.3	97.2		
Avg	84.0	85.9	80.3	90.4	84.0	94.6		

Figure 4. 5: The relationship between the d3ifferent *Moringa oleifera* seed solution extracts and fractions and their related egg mortality percentages

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4.4.2 The larval survivability assay with *Moringa oleifera* seed crude extract and fractions

Figure 4.6 illustrates the mortality levels of *H. contortus* larvae in the larval survivability assay (LSA), where the *M. oleifera* crude extracts and fractions at different concentrations were analysed. This particular assay showed the lowest overall mortality effects against *H. contortus* larvae, where the 5 mg/ml (16.2%) and 10 mg/ml (8.7%) concentration treatment groups of fraction 5 were comparable ($P>0.05$) to that of the negative control group, being the only assay of the study to reveal such a result.

In contrast, fraction 3 was the only treatment group at concentrations of 10 mg/ml and 15 mg/ml that were comparable ($P>0.05$) to that of the positive control group, with mortality levels of 76.9% and 88.7%. Contrary to being comparable to the positive control, the mean mortality level of fraction 3 (71.4%) was significantly lower ($P<0.05$) to that of the positive control, which could be interpreted that specific bioactive molecules have either additive or antagonistic interactions with each other (Vargas-Magañ a *et al.*, 2014), which was evident with fraction 3, as the 5 mg/ml concentration treatment group was significantly ($P<0.05$) lower than the fraction 3 concentration groups 10 mg/ml and 15 mg/ml (48.4% versus 76.9% and 88.7% respectively).

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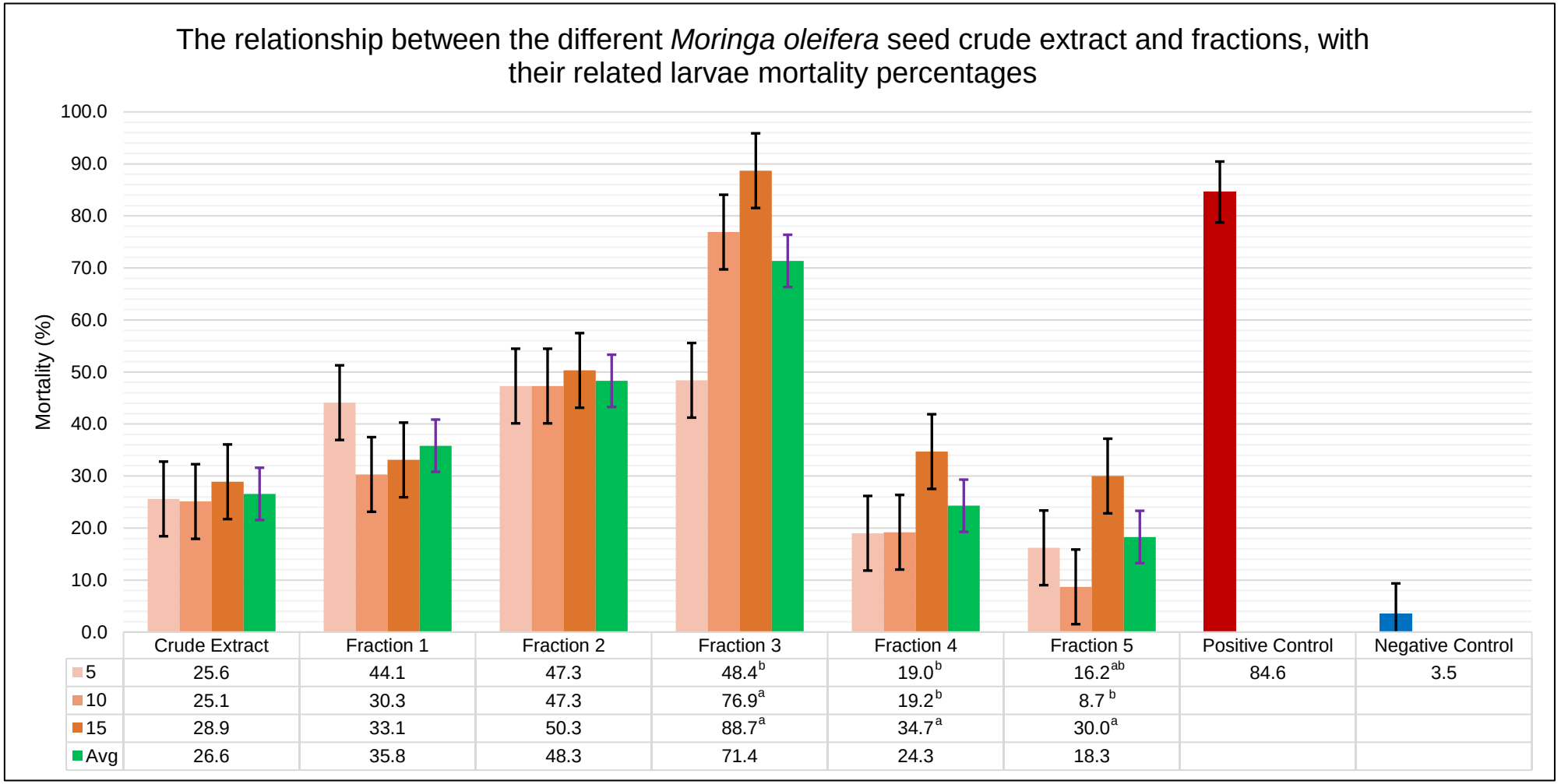

	Crude Extract	Fraction 1	Fraction 2	Fraction 3	Fraction 4	Fraction 5	Positive Control	Negative Control
5	25.6	44.1	47.3	48.4^{b}	19.0^{b}	16.2^{ab}	84.6	3.5
10	25.1	30.3	47.3	76.9^{a}	19.2^{b}	8.7^{b}		
15	28.9	33.1	50.3	88.7^{a}	34.7^{a}	30.0^{a}		
Avg	26.6	35.8	48.3	71.4	24.3	18.3		

Figure 4. 6: The relationship between the different *Moringa oleifera* seed solution concentrations and their related larvae mortality percentages

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4.4.3 Fractionation of *Moringa oleifera* seed crude extract and anthelmintic effect against *Haemonchus contortus* eggs and larvae

Seed fractions had overall lower anthelmintic efficacy against *H. contortus* in contrast to the leaf extracts, with the anthelmintic potency being higher in the EHA versus the LSA, as illustrated in Figures 4.1, 4.2, 4.5 and 4.6. Fraction 5 (F5) had the highest mean mortality percentage in the EHA (94.6%) and was comparable to the mortality levels of the positive control treatment group (P>0.05). Thus, F5 was chosen for UPLSC-UV/QTOF/MS analysis and revealed different secondary phytochemicals illustrated in Table 4.6. The high mortalities could be due to the sesquiterpenoids present (Oliveira *et al.*, 2014), as in the leaf fractions mentioned above. Coumarins, a subgroup of a larger class of flavones, were also identified in fraction 5, and these have been reported to express anthelmintic activity against *H. contortus* (Silva *et al.*, 2019; Mumed *et al.*, 2022). Steroidal alkaloids have not been reported to act against *H. contortus* but do possess potential anthelmintic activity due to reports showing anthelmintic activity against *Schistosoma* sp. (Miranda *et al.*, 2012).

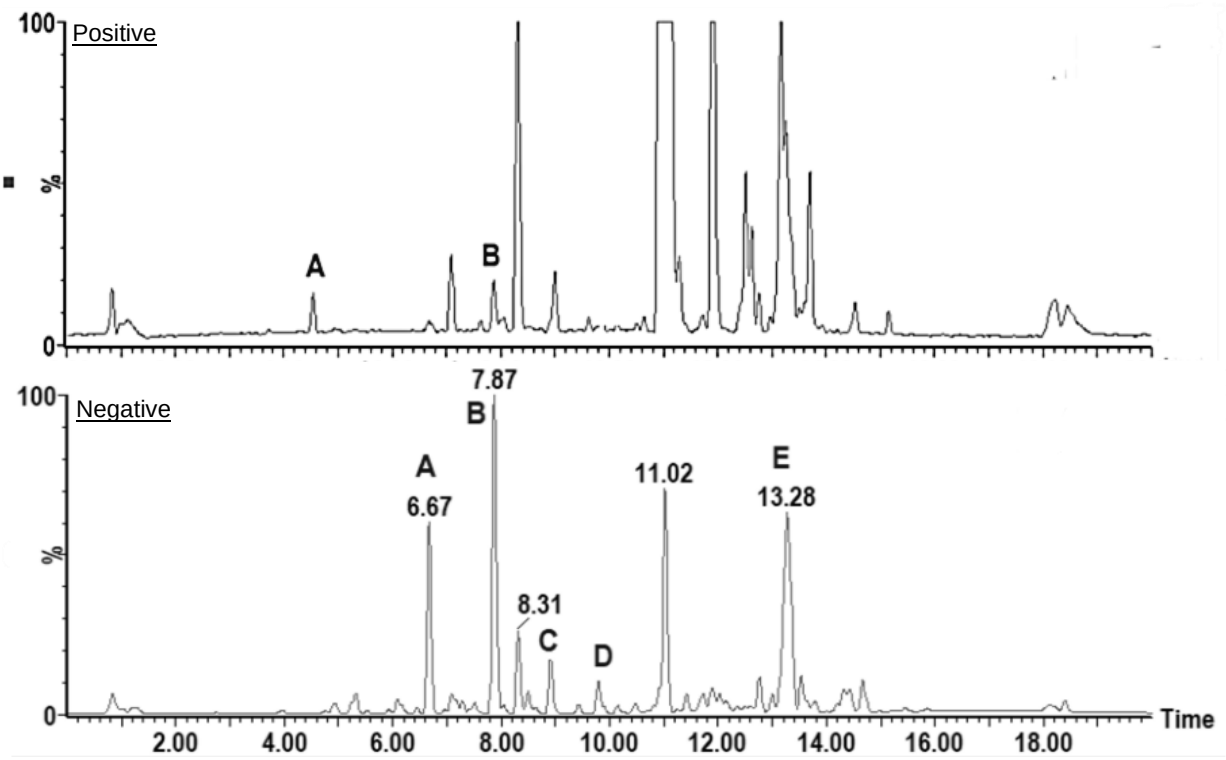

Figure 4. 7: *Moringa oleifera* leaf fraction 5 UPLC-UV-QTOF/MS positive and negative chromatograms

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Table 4.6: *Moringa oleifera* crude seed extract with fraction 5 phytochemical compounds after UPLC-UV-QTOF/MS test

	Compound Name	Time	Compound Class
A^+	Unspecified sesquiterpenoid	4.54	Sesquiterpenoid
B^+	Buxmicrophylline H	7.87	Steroidal alkaloids
A^-	2,5-dimethylhexanedioic acid	6.67	Dicarboxylic acids
B^-	Buxmicrophylline H	7.87	Steroidal alkaloids
C^-	Monoisobutyl adipate	8.90	Wax monoesters
D^-	-	9.79	Flavones / Coumarins
E^-	-	13.28	Octadecanedioic acid derivatives

Fraction 3 of the *M. oleifera* seed extract had the highest average anthelmintic efficacy (71.4%) in the LSA (Figure 4.6) and was chosen for UPLSC-UV/QTOF/MS analysis. It should be noted that this LSA had the poorest anthelmintic efficacy, at 26.6% mean anthelmintic efficacy, even though compounds with reported anthelmintic potential were observed and illustrated in Table 4.7 below. These compounds are different subgroups of terpenoids, with sesquiterpenoids being the most, as discussed by Oliveira *et al.* (2014). Guanine sesquiterpenoids were interestingly only observed in F3 of the *M. oleifera* seed crude extract. Foster *et al.* (2011) mentioned that this class of secondary metabolites possess anthelmintic properties, which could explain the high mean mortality levels of fraction 3 in the EHA and LSA (90.4% and 71.4% respectively).

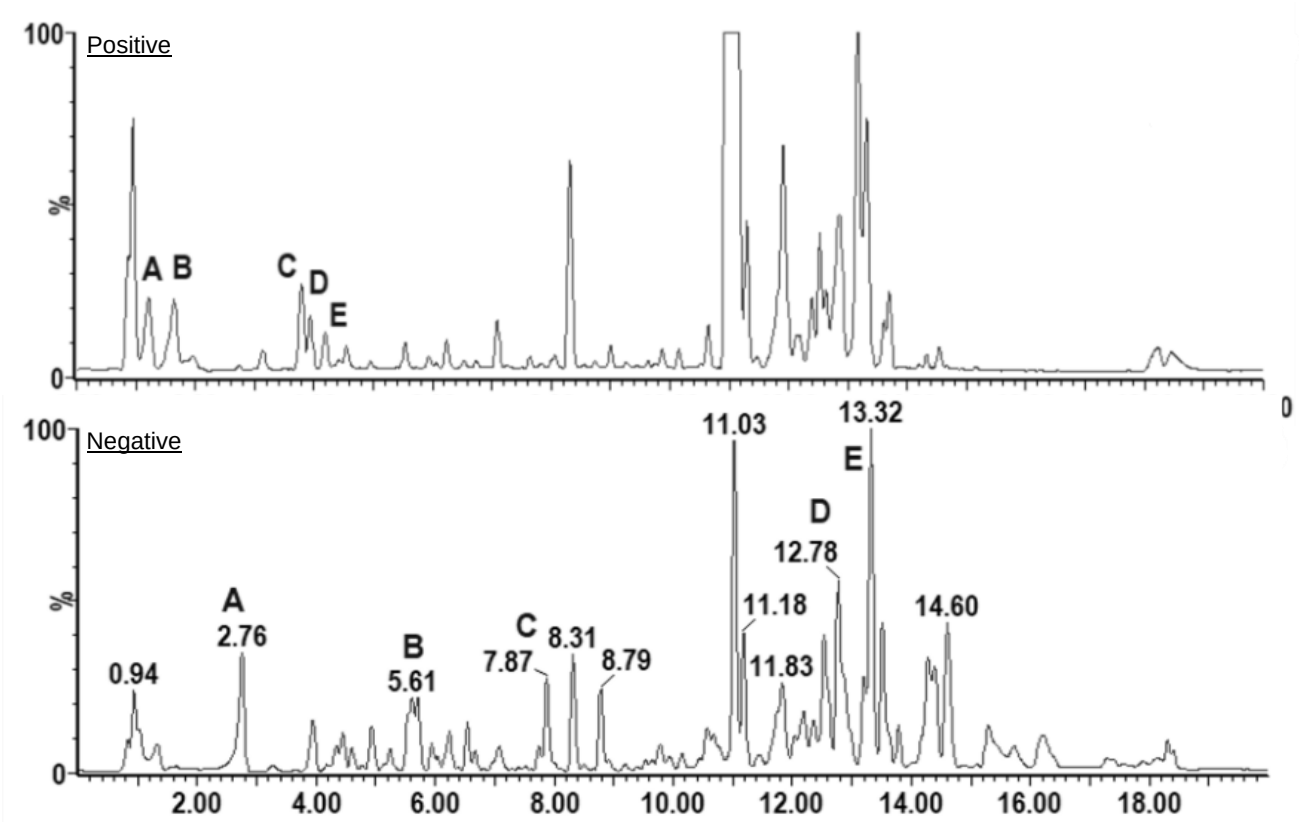

Figure 4. 8: *Moringa oleifera* seed fraction 3 UPLC-UV-QTOF/MS positive and negative chromatograms

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Table 4.7: *Moringa oleifera* crude seed extract with fraction 3 phytochemical compounds after UPLC-UV-QTOF/MS analysis.

	Compound Name	Time	Compound Class
A^+	-	1.22	Benzaldehyde
B^+	Miserotoxin	1.62	Glycoside
C^+	*Sesquiterpenoid 1*	3.77	Aromadendrane sesquiterpenoids
D^+	7-isopropyl-1,1-dimethyl-5,6-dioxo-3,10a-dihydro-2h-phenanthren-2-yl benzoate	3.94	Diterpenoids
E^+	Artanomaloide	4.19	Guaiane sesquiterpenoids
A^-	4-(alpha-L-Rhamnopyranosyloxy) benzyl glucosinolate	2.76	Glucosinolates
B^-	Benzyl beta-primeveroside	5.61	Polysaccharides
C^-	8-ethyl-11,19-dihydroxy-20-[5'-(1-hydroxyethyl)-2,5'-dimethyl-[2,2'-bioxolan]-5-yl]-2,4,6,12,14,18-hexamethyl-9-oxoicosa-10,16-dienoic acid	8.31	Open-chain polyketides
D^-	9,10-Dihydroxystearic acid	12.78	Octadecanoids
E^-	Ricinoleic acid	13.32	Hydroxy fatty acids and Octadecanoids

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Chapter 5: Conclusions & Recommendations

5.1 Conclusions

Numerous studies have explored the anthelmintic properties of *Moringa oleifera*, and this study aimed to add value by confirming that *M. oleifera* has anthelmintic efficacy while contributing new data to support its potential applications in parasite management. The findings of this project suggest that *M. oleifera* is a promising candidate for addressing anthelmintic challenges posed by *H. contortus*, particularly in targeting the egg hatching stage. The results demonstrate the significant anthelmintic potential of *M. oleifera* extracts against *H. contortus*, with the leaf extracts exhibiting higher mortality rates compared to the seed extracts. The highest ovicidal activity was observed at a leaf crude extract concentration of 15 mg/mL, achieving 100% mortality, while the seeds reached a maximum of 95% mortality at 10 mg/ml. The highest larvicidal activity was observed with the *M. oleifera* leaf crude extracts at a concentration of 15 mg/ml with a larval mortality rate of 45.4%, which defines the anthelmintic potential of *M. oleifera* more on the egg life stage of *H. contortus* than the larval life stage.

Analysis of bioactive compounds revealed that several phytochemicals are likely responsible for the observed anthelmintic effects. Key bioactive molecules identified in *M. oleifera* include terpenoids, flavonoids, alkaloids, and tannins. These compounds have been previously documented for their anthelmintic properties against various gastrointestinal nematodes, supporting their role in the efficacy of *M. oleifera* extracts. The compounds identified in the fractions of the crude extracts such as cinnamic acids, sesquiterpenoids, guaiane terpenoids, and menthane monoterpenoids have demonstrated in previous studies to have anthelmintic properties. The presence of these bioactive molecules in the crude extracts may contribute to the observed high mortality rates, particularly in the egg hatch assay (EHA) and larval survivability assay (LSA). Overall, this study highlights *M. oleifera* as a potential viable alternative to conventional anthelmintics, with its various fractions demonstrating significant efficacy against *H. contortus*.

By integrating *M. oleifera* extracts into deworming programs, small-scale sheep farmers in South Africa could reduce dependency on expensive commercial anthelmintics. This locally-available, cost-effective intervention could enhance flock health, productivity, and farmer resilience in resource-limited rural systems.

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5.2 Recommendations

Based on the findings of the project, it is recommended to proceed with further investigations to specify the underlying mechanisms of anthelmintic efficacy of phytochemicals present in *M. oleifera* fractions against *H. contortus*. These in vitro studies should be explicitly designed to assess not only efficacy, but also the consistency of active components across different plant batches, thereby evaluating the practical viability of using whole-plant preparations or standardized extracts. Moreover, clear acknowledgment must be made of regulatory hurdles: in South Africa, any agricultural remedy intended as a parasiticide must comply with Act 36 of 1947 (the Fertilizers, Farm Feeds, Seeds and Remedies Act), which requires efficacy, residue, safety data, and official registration before legal use in livestock. The fractions demonstrating the highest overall mortalities should undergo bioassay-guided fractionation and isolation of bioactive compounds to detect which compounds are responsible for bioactivity and can subsequently be used as chemical markers for quality control of *Moringa* preparations, as chemical variation could influence bioactivity. These fractions should be used to conduct another *in vitro* study, which can provide deeper insights into the specific phytochemical compounds contributing to the reduction in mortality percentages.

Furthermore, to assess the practical applicability and potential external influences on the efficacy of the extracts, conducting an *in vivo* study is essential. Such studies would aid in identifying potential effects of South African environmental factors, including climate, soil composition, and grazing methods, on the plant's phytochemical profile and anthelmintic efficacy. Additionally, since differences in breed physiology may affect outcomes, it is crucial to determine whether various cattle breeds that are frequently found in South Africa react differently to the extracts. Overall, these recommended steps will not only enhance our understanding of the potential of *M. oleifera* potential as an anthelmintic agent but also pave the way for its practical implementation in livestock management strategies.

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