Efficacy of selected plant species against *Meloidogyne incognita* and phytopathogenic microbes infecting tomatoes

Fikile Nelly Makhubu (17407232)

Thesis submitted in fulfilment of the requirements for the degree of

Philosophiae Doctor (PhD)

In the

Phytomedicine Programme, Department of Paraclinical Sciences

Faculty of Veterinary Sciences, University of Pretoria

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Promoter: Prof L.J. McGaw

Co-Promoter: Dr M.C. Khosa

2021

DECLARATION

I, Fikile Nelly Makhubu, hereby declare that the experimental work described in this thesis is my original work (except where the input of others is acknowledged), conducted in the Phytomedicine Programme, Department of Paraclinical Sciences, Faculty of Veterinary Sciences, University of Pretoria, at the Vegetable and Ornamental Plant Campus, ARC-Tropical and Subtropical Crops Campus and at the Faculty of Agriculture and Natural Sciences at the University of Mpumalanga. The work has not been submitted in any other form to any University or academic institution.

……

Fikile Nelly Makhubu

DEDICATION

This work is dedicated to my family, mother Malintja Belina Makhubu and my son Karabo Makhubu for their support throughout my studies.

ACKNOWLEDGEMENTS

I would like to extend my heartfelt gratitude to my supervisor Prof. L.J. McGaw for her guidance, support and continuous encouragement throughout this study. Thank you so much for your dedication towards this project and being a good advisor. To my co-supervisor Dr M.C. Khosa, thank you so much for introducing me to the discipline of Crop Protection with emphasis on Integrated Pest Management and plant-parasitic nematodes. Your guidance, support and contribution in this study is highly appreciated.

I am extremely grateful to Prof. G. Fouche for her assistance in isolation and identifying the isolated compounds described in this thesis; Prof. M.A. Aderogba is thanked for starting the isolation process. I thank Dr K.M. Pofu and Dr Z.P. Dube for their immense support in the glasshouse trials and analysis. I am thankful for Ms S.M. Nkadimeng for being helpful in the cytotoxicity aspect of this work.

Sincere gratitude to Dr B.M. Madikizela and Dr T.E. Ramadwa for their continual support, guidance and words of encouragement throughout the study. Their critical review of this work helped in construction of the thesis and this has helped me to grow from strength to strength.

Thank you to the LC-MS Synapt Facility (Department of Chemistry, University of Pretoria) for chromatography and/ or mass spectrometry services provided by Ms M. Wooding, GC-MS analysis which was performed with the help of Dr Y. Naudé, and NMR analysis which was performed by Dr M. Selepe at the University of Pretoria, Department of Chemistry. Dr C. Van der Westhuyzen, from Council for Scientific and

Industrial Research (CSIR) is thanked for his assistance in analysing my compounds with NMR.

I would also like to extend my gratitude to ARC-Plant Protection Research (ARC-PPR), Roodeplaat, South Africa for providing me with bacterial and fungal strains. The curator Mr. J. Sampson of the University of Pretoria Botanical Garden is thanked for helping with collection of plant material and Ms. M. Nel of the H.G.W.J. Schweickerdt Herbarium for assisting in preparation of voucher specimens. To Dr N. Olivier, Faculty of Natural and Agricultural Sciences from the University of Pretoria, thank you for allowing me to do my phytotoxicity experiments in your laboratory.

I thank the Agricultural Research Council Professional Development Programme (ARC-PDP), Health and Welfare Sector Education and Training Authority (HWSETA) and University of Pretoria Doctoral Support Bursary for financial support of my PhD.

I am thankful to my friends, laboratory and field colleagues in the Phytomedicine Programme, ARC-VOPI, ARC-TSC and students at the University of Mpumalanga for contribution in this study. Your assistance and contribution in various ways helped me to complete this work and I will be forever grateful.

I am thankful to my mother, Mrs. Makhubu Malintja Belina for her sacrifices, love and immense support and believing in me throughout my study life and making sure that my dream becomes a reality. I thank God for blessing me with such a soul, you are a truly “imbokodo”. To my siblings (Nkosana, Mamsy and Jabulani) and my nieces

Tshegofatso, Abongile and nephew Usenathi thank you for amazing love, support and encouragement throughout my studies. You are all my pillar of strength.

I am grateful to my son Karabo Makhubu for being strong and giving me strength throughout my project, allowing me to leave you just to finish this journey, really you are a blessing.

A very special thanks goes to my partner Mr. K. Mallane who has been there for me throughout my project. Thank you for the support, love, and the words of encouragement. Your contribution to making this success is highly appreciated.

Lastly but most importantly, I thank God Almighty for giving me the strength, courage, wisdom and opportunity to undertake this study. Your mercy and love kept me going, you are amazing and indeed you deserve all the glory.

Kea leboha

LIST OF ABBREVIATIONS

Ace	Acetone
AChE	Acetylcholinesterase
APN	Animal-parasitic nematodes
ARC	Agricultural Research Council
BUT	Butanol
CA	Colorimetric assay
DBA	Developmental and behavioural assay
$CDCl_3$	Deuterated chloroform
CFU/ml	Colony forming units/ml
CO_2	Carbon dioxide
CRD	Completely randomized design
DAFF	Department of Agriculture, Forestry and Fisheries
DCM	Dichloromethane
DMSO	Dimethyl sulfoxide
EC_{50}	Effective concentration for 50% of organisms
EHA	Egg hatch activity
EtOAc	Ethyl acetate
FAO	Food and Agricultural Organisation
FLN	Free-living nematodes
GABA	Gamma-amino butyric acid
GC-MS	Gas chromatography-mass spectroscopy
GLT	Ground leaching technology
Hex	Hexane
HIV	Human immunodeficiency virus
H_2O	Water
INT	*p*-iodonitrotetrazolium violet
LC_{50}	Lethal concentration for 50 percent
LDA	Larval development assay
MEM	Minimal essential medium
MeOH	Methanol
MHz	Mega Hertz
MIC	Minimum inhibitory concentration

MLC	Minimum lethal concentration
MS	Mass spectrometry
MTT	3-(4, 5-Dimethylthiazolyl-2)-2,5-diphenyltetrazolium
NaOCl	Sodium hypochlorite
NMR	Nuclear Magnetic Resonance
PCR	Polymerase Chain Reaction
PPN	Plant-parasitic nematodes
QTOF	Quadrupole Time of Flight
RCBD	Randomized-complete block design
RKN	Root-Knot Nematode
SCAR	Sequence Characterised Amplified Region
SI	Selectivity index
TLC	Thin layer chromatography
TSC	Tropical and Subtropical Crops
UP-LC	Ultra Performance Liquid Chromatography
UV	Ultraviolet
WHO	World Health Organization

RESEARCH OUTPUTS

Conference presentation

Makhubu, F.N., Khosa, M.C., and McGaw, L.J. 2018: Preliminary screening of selected South African medicinal plants for the control of root-knot nematodes, *Meloidogyne incognita* (Poster). Faculty Day, 23 August 2018 held at the University of Pretoria, Faculty of Veterinary Science, Tshwane, Gauteng, South Africa.

Makhubu, F.N., Khosa, M.C., and McGaw, L.J. 2019. Comparison of efficacy of five South African plants against free-living *Caenorhabditis elegans* and plant-parasitic *Meloidogyne incognita* and *in vitro* safety evaluation (Oral). 45th SAAB, AMA & SASSB Joint Congress, 8–11 January 2019, Johannesburg, Gauteng, South Africa.

Makhubu, F.N., Khosa, M.C., and McGaw, L.J. 2019. Activity of three South African plants on phytopathogenic bacteria and fungi of tomatoes and chemical profiling of the extracts. (Poster). Faculty Day, 22 August 2019 held at the University of Pretoria, Faculty of Veterinary Science, Tshwane, Gauteng, South Africa.

Makhubu, F.N., Khosa, M.C., and McGaw, L.J. 2019. Can plants with good anthelmintic activity against free-living and animal-parasitic nematodes be effective against plant-parasitic helminths? (Poster) 67th International Congress and Annual Meeting of the Society for Medicinal Plant and Natural Product Research (GA), 1-5 September 2019, Innsbruck, Austria.

Makhubu, F.N., Khosa, M.C., and McGaw, L.J. 2019. Activity of three South African plants on phytopathogenic bacteria and fungi of tomatoes and chemical profiling of the

extracts. (Poster). 67th International Congress and Annual Meeting of the Society or Medicinal Plant and Natural Product Research (GA), 1-5 September 2019, Innsbruck, Austria.

Makhubu, F.N., Khosa, M.C., and McGaw, L.J. 2020. *In vitro* investigation of *Leonotis leonurus* and *Clausena anisata* extracts on the egg hatch and motility of *Meloidogyne incognita* second-stage juveniles. (Oral). 46th South African Association of Botanists (SAAB) Annual Conference, 7-10 January 2020, Qwaqwa, Free State Province, South Africa.

Makhubu, F.N., Khosa, M.C., and McGaw, L.J. 2020. Efficacy of *Leonotis leonurus* against phytopathogens nematodes infecting tomatoes. (Poster presentation). Virtual Faculty Day, 20 November 2020 held at the University of Pretoria, Faculty of Veterinary Science, Tshwane, Gauteng, South Africa.

Publications

Makhubu, FN., Khosa, M.C., McGaw, L.J. 2020. South African plants with nematicidal activity against root-knot nematodes: a review. South African Journal of Botany, 139, 183-191. https://doi.org/10.1016/j.sajb.2021.02.010

Makhubu, F.N., Khosa, M.C. and McGaw, L.J., 2019. Can plants with good anthelmintic activity against free-living and animal-parasitic nematodes be effective against plant-parasitic nematodes? Planta Medica, 85(18), pp.PV-10. (Abstract)

Makhubu, F.N., Khosa, M.C. and McGaw, L.J., 2019. Activity of three South African plants on phytopathogenic bacteria and fungi of tomatoes and chemical profiling of the extracts. Planta Medica, 85(18), pp.P-235. (Abstract)

Manuscripts under review and to be submitted

Makhubu, FN., Khosa, M.C., McGaw, L.J. 2021. Investigating the potential of South African plants as crop protective agents against soilborne pathogens infecting tomatoes (Submitted to Journal of Phytopathology)

Makhubu F.N., Nkadimeng, S.M., Fouche, G., Khosa, M.C., McGaw, L.J. 2021. Comparative anthelmintic activity against free-living and animal-parasitic nematodes and phytochemical profiling of South African selected plants. BMC Veterinary Research (to be submitted)

Makhubu F.N., Nkadimeng, S.M., Fouche, G., Khosa, M.C., McGaw, L.J. 2021. Isolation and characterisation of nematicidal compounds from *Leonotis leonurus* acetone leaf extract. Journal of Ethnopharmacology (to be submitted)

Makhubu, F.N., Pofu, K.M., Dube, Z.P., Khosa, M.C., McGaw, L.J. 2021. Efficacy *of Leonotis leonurus, Clausena anisata* and *Lantana rugosa* on growth of tomato seedlings and suppression of *Meloidogyne incognita*. Crop Protection. (to be submitted)

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Veterinary Science

Research Ethics Committee

Project Title	**Screening for efficacy of selected plant species against root-knot nematodes (Meloidogyne spp).**
Project Number	**REC115-19**
Researcher / Principal Investigator	**Miss FN Makhubu**

DIissertation / Thesis submitted for	**Doctoral**

Supervisor	**Prof LJ McGaw**

APPROVED	Date: **2019-07-02**
CHAIRMAN: UP Research Ethics Committee	Signature:

Office of the Chairman: Research Ethics Committee
Room 2-24, Pathology Building, Onderstepoort
University of Pretoria, Private Bag X04
Onderstepoort 0110, South Africa
Tel +27 (0)12 529 8052
Email
www.up.ac.za

ABSTRACT

Soilborne pathogens are economically important, causing great losses in agricultural production globally. Nematodes, fungi, bacteria and viruses are microscopic, destructive pathogens which are extremely difficult to control. Farmers depend on synthetic pesticides for quick and effective control of pests and diseases. However, the majority of nematicides have been banned due to environmental problems resulting from their use and this has awakened interest in finding alternative methods of nematode and other plant pathogen control. Several indigenous plants have been identified as having potential anthelmintic efficacy in managing gastrointestinal infections in small ruminants and some of these have also been found to be effective in killing nematodes infecting crops.

In the present study, extracts of plants previously reported to have anthelmintic activity against free-living *Caenorhabditis elegans* and animal parasitic *Haemonchus contortus* nematodes were screened for their efficacy against the root-knot nematode species *Meloidogyne incognita*. Activity of the selected plants was confirmed against *C. elegans* and *H. contortus.* For *in vitro* safety determination, cytotoxicity tests on *Leonotis leonurus, Clausena anisata* and *Lantana rugosa* was conducted against Vero African green monkey kidney cells. Chemical profiling was performed using gas chromatography-mass spectrometry (GC-MS). The plants were further investigated for their ability to inhibit the growth of interrelated phytopathogens infecting tomatoes, including five pathogenic bacterial species: *Clavibacter michiganensis* subsp. *michiganensis*, *Xanthomonas vesicatoria*, *X. perforans, Ralstonia solanacearum* and *R. pseudosolanacearum* and one fungal strain (*Fusarium oxysporum* f.sp. *lycopersici*). Bioactive compounds were isolated from the acetone leaf extract of *L. leonurus* using

bioassay-guided fractionation. *Clausena anisata, L. rugosa* and *L. leonurus* water extracts were subjected to *in vitro* phytotoxicity tests on tomato seedlings, and an *in vivo* glasshouse trial against *M. incognita* was also conducted with the powdered material (dried leaves) of *L. leonurus* and *C. anisata*.

All plant extracts and fractions investigated had good anthelmintic activity against free-living and animal-parasitic nematodes. Activity of the plant species *Acokanthera oppositifolia*, *Searsia lancea*, *Cotyledon orbiculata*, *Hippobromus pauciflorus* and *Lantana rugosa* was reported for the first time against *Haemonchus contortus.* The phytochemicals detected in the extracts contribute to the activity of the plants reported, as some of these compounds were previously reported to have antibacterial, insecticidal and nematicidal properties. Cytotoxicity results indicated that *C. anisata* and *L. leonurus* extracts were relatively non-toxic to Vero cells compared to the positive control doxorubicin (LC_{50} = 0.0133 mg/mL). *Lantana rugosa* extracts were highly toxic with LC_{50} values less than 0.0075 mg/mL, the lowest concentration tested.

When screening the selected plants for activity against *M. incognita* second-stage juveniles (J2) at the highest concentration of 1 mg/ml, results indicated that all selected non-crop plant species had potential in managing root-knot nematodes, but the most promising activity was observed with *C. anisata*, *L. rugosa* and *L. leonurus* extracts. The three plants were further evaluated in terms of motility against J2s as well as J2 ~~egg~~ hatch inhibition. Only the *L. leonurus* water extract showed good dose-related activity in inhibiting motility of *M. incognita* J2s. *Clausena anisata* extracts had weak activity in the motility assay but both plants had good inhibitory activities against J2 hatching.

In the antimicrobial assay, acetone extracts of *Leucosidea sericea* and *Searsia lancea* demonstrated interesting antibacterial activity against a broad range of tested bacteria with MIC values ranging from 19.5 to 97.5 µg/mL. None of the extracts was active against *Fusarium oxysporum* f. sp. *lycopersici* except for the acetone extract of *Cotyledon orbiculata* and *L. leonurus* water extract, which inhibited the growth of *F. oxysporum* f.sp. *lycopersici* at 39 µg/mL and 97.5 µg/mL after 24 h respectively. *Clausena anisata* and *L. leonurus* fractions obtained from liquid/liquid partitioning indicated poor activity against all tested phytopathogens, except for the *L. rugosa* fractions which had good to moderate activity against most bacterial pathogens with MIC values ranging from 78 to 156 µg/mL.

Bioassay-guided fractionation was used to isolate nematicidal compounds from the dichloromethane fraction of *Leonotis leonurus* using *Caenorhabditis elegans* as the test organism. This led to isolation of leoleorin C, and other compounds which could not be identified due to insufficient time and purity. Leoleorin C was previously isolated from the plant, but activity against selected organisms and the *in vitro* lack of cytotoxicity is reported for the first time in this study. Leoleorin C had moderate activity against *C. elegans* but was not active against *M. incognita*. The compound was not active against the tested bacterial phytopathogens, but promising activity was observed in the bioautography assay against *Clavibacter michiganensis* subsp. *michiganensis*, causal organism of bacterial canker in tomatoes. The plant was not toxic to the Vero cells at the highest concentration of 1 mg/mL.

Leonotis leonurus, *C. anisata* and *L. rugosa* water extracts were not phytotoxic to the growth of tomato seedlings at all tested concentrations (highest concentration of 10

mg/mL) under *in vitro* conditions. Lower extract concentrations of all the selected non-crop plant species stimulated germination, whereas high concentrations had inhibitory effects, except for the *C. anisata* extract. In the treatment of tomato plants under *in vivo* conditions, *M. incognita* infection did not negatively affect the growth parameters assessed, namely stem height, wet shoot mass, dry shoot mass, number of flowers, stem diameter, fruit number and fruit mass. The plants had the ability to reduce gall index, number of eggs and J2 when compared to the untreated control of tomato plants. There was a lack of phytotoxic and fertilizer effect on tomato plants at the applied rates on growth parameters since activity was comparable to the untreated control. The low effect demonstrated by the plants might be due to low quantities of active phytochemicals present. *Leonotis leonurus* and *C. anisata* were not effective in the form of powder as nematicidal preparations in the glasshouse trials.

LIST OF FIGURES

LIST OF TABLES

TABLE OF CONTENTS

CHAPTER 1

Introduction

1.1 Background and motivation

Agricultural production is expected to be in high demand due to the increase in human population which is estimated by the Food and Agricultural Organisation (FAO) to grow to almost 10 billion by 2050 (FAO, 2017). According to Agrios (2005), an approximate 36% decrease in crop production may be attributed to a combination of plant disease, insects and weeds with plant disease alone causing a 14% reduction in yield. Nematodes are one of the most important economic pests affecting productivity in agriculture, with plant-parasitic nematodes (PPN) representing the most significant pests (Nicol et al., 2011). These nematodes directly target the roots of major crops, thus preventing the translocation of water and nutrient uptake, resulting in reduction of agronomic performance, overall quality and quantity (Bernard et al., 2017). The root-knot nematode (RKN) *Meloidogyne incognita* (Kofoid and White) Chitwood is considered to be one of the most important PPN species affecting the quantity and quality of production of many annual and perennial crops (Pavaraj et al., 2012).

Nematodes that infect animals cause great losses in animal production worldwide. Gastrointestinal nematodes in small ruminants are responsible for decrease of production due to stock mortality, weight loss and poor production (Perry and Randolph, 1999). *Trichostrongylus colubriformis* and *Haemonchus contortus* remain the most common nematodes causing significant loss of production in ruminants (Horak and Ursula, 2004). *Haemonchus contortus* is one of the most important

parasites infecting ruminants worldwide, causing substantial losses to the livestock industry estimated at USD10 billion dollars per annum (Waller and Chandrawathani, 2005; Roeber et al., 2013).

Plants are highly susceptible to infection caused by other pathogens such as bacteria and fungi following wounding of a root or below-ground part as a result of nematode infection (Williamson and Gleason, 2003; Manzanilla-López and Starr, 2009). Several soil-borne pathogens in the fungal and bacterial kingdom have been reported to cause destruction of large fields of agricultural crops and also great economic losses worldwide. They include species of *Alternaria*, *Clavibacter, Fusarium*, *Ralstonia*, and *Xanthomonas* (Yabuuchi et al., 1995 cited by Li and Dong, 2013). These pathogens have a synergistic relationship with nematodes. For example, Fusarium wilt caused by *Fusarium oxysporum* f. sp. *lycopersici* is an important disease of tomato. It causes deterioration in the root and the basal stem and results in wilting of vegetable plants (Ignjatov et al., 2012).

Farmers depend on synthetic pesticides for quick and effective control of pests and diseases (Gahukar, 2018). Nematicides such as methyl bromide, dibromo chloropropane, ethylene dibromide and aldicarb (Temik) have been banned in various parts of the world due to their environmental and human toxicity (Onkendi et al., 2014). Current control strategies against *H. contortus* primarily rely on repeated anthelmintic treatments. However, the excessive use of such drugs has led to widespread resistance in these nematodes to most classes of anthelmintics (Kaplan and Vidyashankar, 2012), making management more difficult. The problems with currently-used nematicides and anthelmintic drugs have raised the need to find alternative, low

input, cost-effective and environmentally-friendly nematode strategies to alleviate the pest problem of RKN and deleterious effects of helminths.

Several indigenous plants have been identified for their nematicidal action on root-knot nematodes (Sivakumar and Gunasekaran, 2011) and as anthelmintics to manage gastrointestinal infections in small ruminants (Waterman et al., 2010; Simon et al., 2012; Adamu et al., 2013). In some studies, other plants have been reported to have anthelmintic activity as well as nematicidal properties against PPN. In South Africa, studies on the use of medicinal plants for anthelmintic activity in animals have increased, but investigation of the use of plants for crop protection against PPN has been neglected. In this regard, the South African flora needs to be explored for managing diseases of plants caused by parasitic nematodes, and they can also potentially be used against parasites infecting animals and humans.

1.2 Problem statement

Root-knot nematodes are a major constraint in agricultural production affecting the quality and quantity of crops. Most nematicides (Class 1) have been banned due to environmental problems resulting from their use. The investigation of plants with nematicidal activity is a possible solution to the above-mentioned problem.

1.3 Hypothesis

Plants are a useful source of nematicidal compounds, and activity of plant extracts and isolated compounds against free-living nematodes (FLN) (*Caenorhabditis elegans*) correlates with activity against plant (*Meloidogyne incognita*) and animal-parasitic nematodes (APN) (*Haemonchus contortus*).

1.4 Aim

The aim of the proposed study was to determine the anthelmintic efficacy of selected non-crop plant species in an effort to identify those compounds/phytochemicals with nematicidal activity against RKN. The aim incorporates an investigation of the correlation of activity against FLN and APN with activity against PPN.

1.5 Objectives

The objectives of the study are:

- To select plant species through literature survey with good activity against the free-living nematode *Caenorhabditis elegans* and the animal-parasitic *Haemonchus contortus*
- To compile a review on South African plants with nematicidal activity against root-knot nematodes
- To evaluate the nematicidal activity of selected plant extracts against *Meloidogyne incognita* and to confirm their anthelmintic activity against *Haemonchus contortus* and *Caenorhabditis elegans*
- To determine the cytotoxicity of the most active extracts using the methyl tetrazolium (MTT) assay against Vero kidney cells
- To fractionate the most active plants with nematicidal activity against *M. incognita* and to test fractions for activity against *C. elegans*, *H. contortus* and *M. incognita* as well as for cytotoxicity
- To determine the minimum inhibitory concentration of the selected plants against a panel of phytopathogenic bacteria and fungi of the tomatoes and to profile chemical constituents of active plants

- To isolate compounds using bioassay-guided fractionation with *C. elegans* as the test organism
- To test the isolated phytochemicals *in vitro* for activity against *M. incognita*, phytopathogenic bacteria and fungi, and for cytotoxicity
- To test the most active extracts for phytotoxicity against tomato seedlings
- To determine the nematicidal potential of selected active plant species against *Meloidogyne incognita* infecting tomatoes *in vivo* under glasshouse conditions

CHAPTER 2

Literature review

Part of this literature review has been published in South African Journal of Botany (focusing on reviewing South African plants with nematicidal activity against root-knot nematodes). Selection of plants for the current study was done through intensive literature survey for plant species with reported anthelmintic activity against the animal parasitic *Haemonchus contortus* and the free-living nematode *Caenorhabditis elegans*.

2.1 Nematodes

Nematodes are multicellular, unsegmented, soft-bodied worms belonging to the phylum Nematoda (Ali et al., 2013; Blaxter and Koutsovoulos, 2015). They can be found in almost any type of environment and include both parasitic and free-living species. Nematodes infect various types of organisms including plants, insects, animals and humans. Free-living nematodes inhabit marine and freshwater environments, as well as the soils and sediments of all of the various types of land biomes (Bailey, 2020). Nematodes that infect plants include genera of *Aphelenchoides, Helicotylenchus, Heterodera, Pratylenchus, Globodera, Meloidogyne, Rotylenchulus, Tylenchorhynchus* and *Scutellonema* to mention a few. They parasitize major crops such as maize, soybean, sugarcane, carrot, granadilla, ginger, guava, wheat, sunflower, bean, tomato, potato, apple, canola, grapevine, grain sorghum, groundnut and others (Fourie et al., 2017) Genera of parasitic nematodes that infect animals include *Haemonchus*, *Teladorsagia*, *Trichostrongylus*,

Oesophagostomum, *Cooperia*, *Nematodirus*, *Ostertagia*, *Cooperia*, *Cyathostoma*, *Cylicostephanus* and *Cylicocyclus*. These species parasitize goats, cattle, sheep and horse (Silvestre and Cabaret, 2004). Nematodes cause a major reduction in agricultural production.

2.1.1 Plant-parasitic nematodes

Plant-parasitic nematodes (PPN) represent the most important pathogens in agricultural production. As reported by Decraemer and Hunt (2006) cited by Jones et al. (2013), there are over 4 100 species of PPN. According to Williamson and Gleason (2003), all PPN are obligately parasitic, feeding exclusively on the cytoplasm of living plant cells. The most economically important groups of nematodes are the sedentary endoparasites, which include the genera *Heterodera* and *Globodera* (cyst nematodes) and *Meloidogyne* (Jones et al., 2013).

2.1.2 How do nematodes infect plants?

Root-knot nematodes (RKN) are sedentary endoparasites feeding at one site on enlarged and modified cells and represent the most advanced and successful type of parasitism; they are biotrophic and induce profound changes in the roots/other below-ground parts of their host as they feed (Andrés et al., 2012). The genus *Meloidogyne* is comprised of approximately 100 species (Jones et al., 2013) and these pathogens have a variety of hosts such as vegetables, grasses, fruits and weeds (Nicol et al., 2011). RKN have six distinct life-stages including the egg, first stage juvenile (J1), second stage juvenile (J2), third stage juvenile (J3), fourth stage juvenile (J4) and an adult stage. Juvenile *Meloidogyne* parasites hatch from eggs as vermiform second-stages (J2), which incorporates the first moult which occurs within the egg. These

newly hatched J2 have a short free-living stage in the rhizosphere of the host plants. They may re-invade the host plants of their parent or migrate through the soil to find a new host (Agrios, 2005). The J2 invade the root elongation region and migrate in the root until they become sedentary **(Figure 2.1)**. Wyss and Grundler (1992) reported that once J2 juveniles are inside the roots, they migrate inter-cellularly through the vascular cylinder by separating cells at the middle lamella. After migration to these tissues, successful parasitism by RKN is dependent upon the formation of giant cells. RKN J2 feed from the giant cells for 10-12 days, then cease feeding and moult three times (J2 to fourth-stage juveniles: J4) over the next two days, and eventually become adults. Third-stage juveniles (J3) and J4 stages lack a functional stylet and do not feed (Askary, 2008). Adult females are sedentary for the rest of their life, while males, if present, migrate out of the root to fertilize females (Agrios, 2005). Matured female adults in the roots become gravid and erumpent, and root cells increase in size and number causing the distinctive root gall. As stated by Finley (1981), the life span of an adult female may extend to three months and hundreds of eggs may be produced. Females can continue laying eggs even after harvesting of aerial parts of the plants and thus the survival stage between crops is within the eggs, and the J2 are able to survive in anhydrobiotic stages. Once embryogenesis is completed and environmental conditions are favourable, a second generation of J2 will hatch from the eggs and invade susceptible below-ground plant material (Agrios, 2005).

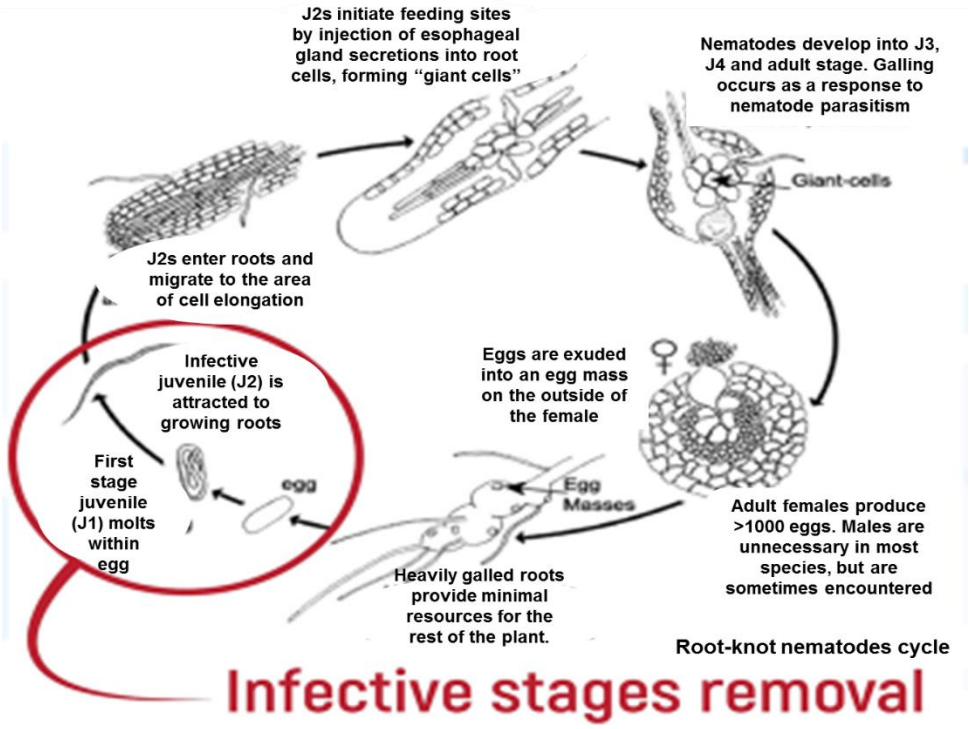


Figure 2.1: Root-knot nematodes life cycle (Adopted from Singh and Phulera, 2015)

2.1.3 Economic importance of root-knot nematodes

More than 2 000 plant species are susceptible to *Meloidogyne* spp. infestation (Agrios,1997 cited by Tranier et al., 2014). *Meloidogyne incognita* (Kofoid and White) Chitwood, *M. javanica* (Treub) Chitwood, *M. arenaria* (Neal) Chitwood, and *M. hapla* Chitwood are among the major agronomically important root-knot nematode species responsible for more than 95% infestation in crops (Sasser et al., 1983; Moens et al., 2009; Khalil, 2014). *Meloidogyne incognita* and *M. javanica* are the most important of the PPN, infecting almost all cultivated plants throughout the world and their impact on yield has been estimated to amount to billions of dollars and euros annually (Blaxter et al., 1998; Bleve-Zacheo et al., 2007). It has been estimated that a worldwide crop loss of 14% is due to *Meloidogyne* spp. which is a concern to producers (Mitkowski and Abawi, 2003; Sahebani and Hadavi, 2008; Collange et al., 2011). In South Africa,

about 14 *Meloidogyne* spp. have been identified to infect various crops (**Table 2.1**). These include the species *M. acronea, M. arenaria, M. chitwoodi, M. enterolobii, M. ethiopica, M. fallax, M. graminicola, M. hapla, M. hispanica, M. incognita, M. javanica, M. kikuyensis, M. partityla* and *M. vandervegte* (Onkendi et al., 2014).

Table 2.1: *Meloidogyne* species reported in South Africa and crops they affect (Adapted from Onkendi et al., 2014)

S/No.	*Meloidogyne* spp.	Crops affected
1.	*M. acronea*	Cotton, pigeon pea, sorghum, millet, grasses, pea, bulrush, okra, potato and tomato
2.	*M. arenaria*	Date palm, peach, potato, tobacco, tea, carrot, cucumber, lettuce, tomato, cotton, aubergine, banana, soybean, pineapple, pyrethrum, okra, pepper, papaya, cowpea, velvet bean
3.	*M. chitwoodi*	Potato, groundnut, wheat and cassava
4.	*M. ethiopica*	Bean, tomato, pepper, cabbage, pumpkin, carrot and tobacco, pineapple, home gardens, natural veld, macadamia, black wattle (*Acacia mearnsii*), potato and soybean,
5.	*M. enterolobii* (= *M. mayaguensis*)	Green pepper, guava and potato
6.	*M. fallax*	Groundnut and tomato
7.	*M. graminicola*	Grass *Paspalum* spp.
8.	*M. hapla*	Potato, groundnut, date palm, native plants and numerous crops
9.	*M. hispanica*	Granadilla, sugarcane, Ficus tree (*Ficus* spp.), ornamental crops and grapevine
10.	*M. incognita*	Potato, grapevine, tomato, soybean, maize, date palm, coconut, tobacco, cowpea, papaya, upland rice, cauliflower, pepper, okra, aubergine, cabbage, onion, watermelon, African spinach, mango, citrus, banana, guava and numerous crops
11.	*M. javanica*	Sugarcane, banana, potato, tobacco, date palm, upland rice, sweet potato, broad bean, celery, aubergine, cabbage, tomato, buchu (*Agathosma betulina*) and numerous crops, cassava and yam
12.	*M. kikuyensis*	Kikuyu grass and sugarcane
13.	*M. partityla*	Pecan and walnut
14.	*M. vandervegtei*	Unidentified woody plant from coastal forest

2.1.4 Symptoms of nematode infection

Plants infested with RKN include symptoms of chlorosis and wilting **(Figure 2.2 b)**, galling of roots and tubers/ other below-ground parts, stunted growth, root lesions and yield loss, and these signs are similar to those of nutrient deficiencies, in particular nitrogen deficiency (Osei et al., 2011; Nguyen et al., 2018). Root galling **(Figure 2.2 d)** is an apparent sign of *Meloidogyne* infection and can easily be diagnosed by farmers (Collange et al., 2011). The induced gall formed in the roots following nematode infection eventually impedes normal uptake of water and nutrients, and it also facilitates infection with some soilborne phytopathogens which may enter the

xylem and disrupt the movement of water, thus causing extensive damage to the crop (Jahr et al., 1999; Bird and Kaloshian, 2003).

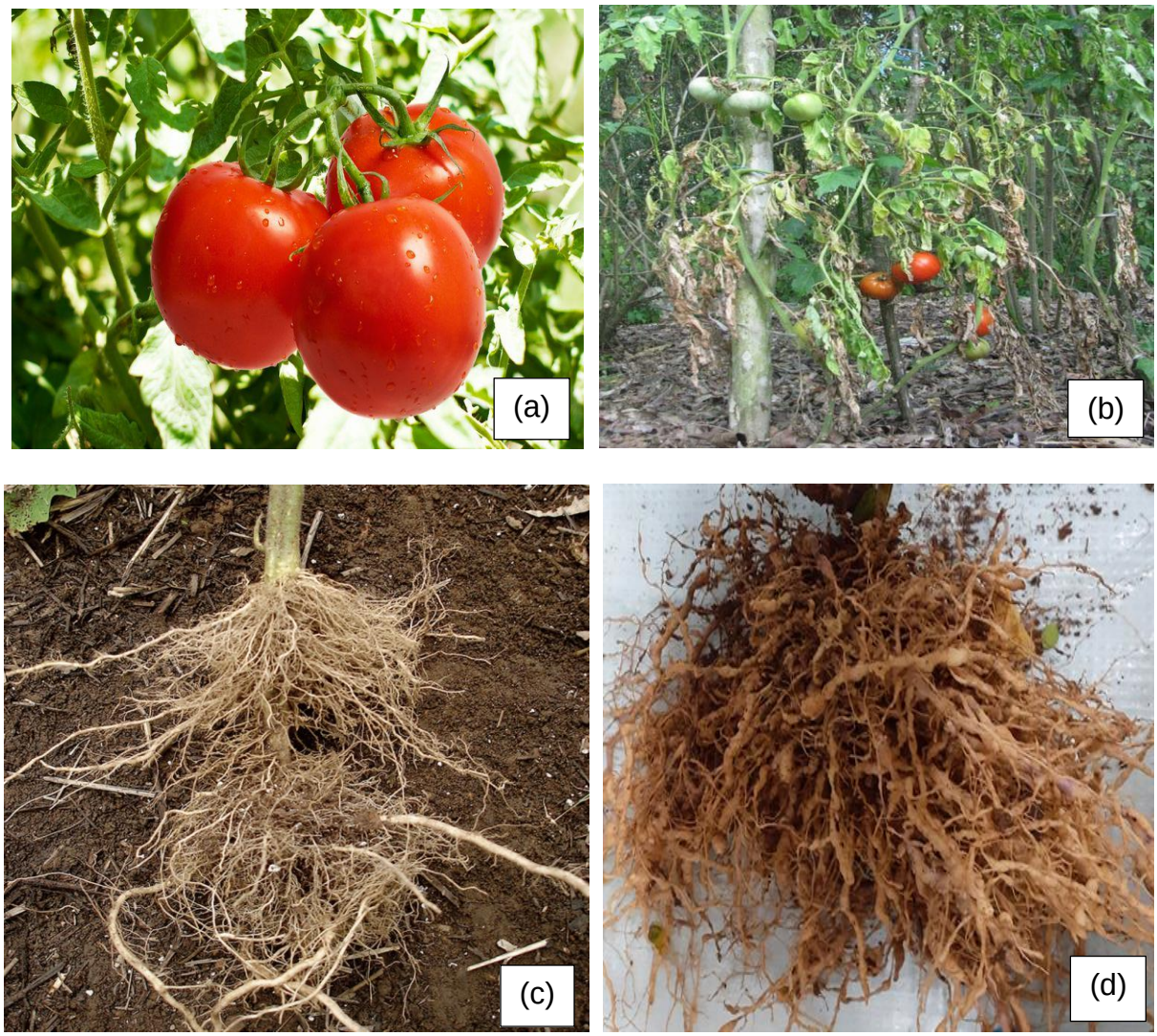


Figure 2.2: Solanaceae plant (*Solanum lycopersicum*) showing healthy plant and root (a) (McFadden, 2021) and (c) (Bonnie, 2021), infested plants (b) (Conrad's, 2012) with galled roots (d) (Photos by FN Makhubu)

2.1.5 Tomato (*Solanum lycopersicum* L.) as important host

The genus *Solanum* is one of the largest genera of angiosperms belonging to the family Solanaceae, originating from the Americas (Kimura and Sinha, 2008; Dari et al., 2016). As reported by Kaunda and Zhang (2019), the *Solanum* genus comprises

about 2 000 species distributed across subtropical and tropical regions including Africa, Asia, America, India and Australia. According to the Food and Agricultural Organization (FAO), tomato is ranked as the first vegetable with respect to world vegetable production and accounts for 14% of world vegetable production (US$ 1.6 billion market value) (FAO, 2010). In South Africa, it is the second most important cultivated vegetable after potato (FAO, 2016) and the Department of Agriculture, Forestry and Fisheries postulated that it alone constitutes close to 17% of the total gross value of vegetable production in 2018 (DAFF, 2019). Tomato are produced in all South African provinces, with Limpopo province being the major production area with 3 590 ha under cultivation (Northern Lowveld at 2 700 ha and far Northern areas of Limpopo at 890 ha), followed by Onderberg area of Mpumalanga province with 770 ha and the border area of the Eastern Cape province at 450 ha in 2018 (DAFF, 2015; DAFF, 2019). The per capita consumption of tomatoes in South Africa is 12 kg per annum and the demand is highly influenced by population growth, urbanization, per capita income and the income elasticity of demand (DAFF, 2019). The South African variation in climate allows the planting and production of good quality fresh tomatoes in open fields in various parts of the country all year. Production is very limited in the winter months and tomatoes can only be produced in frost–free areas during winter or under protection like tunnels (DAFF, 2015).

Tomato is used in many meals, either cooked or uncooked as salads. It can be processed into juice, soup, ketchup, puree, paste or powder (Dari et al., 2016). It is a rich source of vitamins A and C, folic acid, α-lipoic acid, lycopene, choline, β-carotene, and lutein (Afzal et al., 2009). The high amounts of these macronutrients are associated with health benefits, such as the antioxidant properties of lycopene which

reduces the risks associated with several cancers and neurodegenerative diseases as reported by Giovannucci (1999) cited in Seid et al. (2015). On the other hand vitamin A is required for normal functioning of the immune system and for eye health (ARC, 2013). Tomato has been bred to improve productivity, fruit quality and resistance to both biotic and abiotic stresses (Kimura and Sinha, 2008). Reports on *Meloidogyne* spp. infecting tomato plants date back to the end of the 19th century and tomato cultivars have different degrees of susceptibility towards different *Meloidogyne* spp. A tomato cultivar that is susceptible to one population may be moderately resistant to another population of the same species (Seid et al., 2015).

2.2 Control strategies for root-knot nematodes

Methods for controlling PPN that are adopted in most parts of Africa are categorized as chemical, biological or cultural practices. These methods are either practiced singly or in combination to achieve desired results (Onkendi et al., 2014). These practices have been tested in many cases and were found to be effective in reducing various nematode populations, however these methods are genus and species specific (McDonald and Nicol, 2005). Perry and Ploeg (2010) defined methods such as fallowing, crop rotation and soil solarisation as being effective for over a period of a year and that these methods reduce nematode densities only in the top foot or so of the soil. Farmers depend on synthetic pesticides for quick and effective reduction in pest and disease incidence (Gahukar, 2018). Chemicals used, including modes of action, and organic amendments (mainly plants) as control strategies for controlling PPNs are discussed below.

2.2.1 Chemical control

Nematicides are formulated chemicals that can be applied either as pre-planting chemicals, fumigants or as contact nematicides for nematode control (Strajnar and Sirca, 2011). Fumigant nematicides were used previously mainly as pre-planting treatments to lower initial population (Pi) density of nematodes due to their high levels of phytotoxicity, and non-fumigants were mainly used as both pre-emergent and post-emergent nematicides (Chitwood, 2002). Fumigant nematicides (3-dichloropropene, ethylene dibromide, 1,3-D plus chloropicrin, metam potassium, metam sodium and methyl bromide/chloropicrin) have low molecular weight and occur as gas or liquids. As they volatilize, the gas diffuses through the space between soil while the liquid vaporises at ambient temperatures and moves through air spaces in soil particles. When gas and vapour are applied to the soil, they may penetrate deeply where they can have a broad spectrum of biocidal activity (killing fungi, bacteria or even seeds) and the nematodes living in these spaces are killed (Spurr Jr, 1985).

The non-fumigants (carbamates or organophosphates) are formulated as granules while some are available as liquids for spraying on soil or foliage. Non-fumigants move by percolation in soil water, they do not move deeply in soil, have a narrower spectrum of biocidal activity and are non-phytotoxic at suggested use rates and are active at lower dosages (Spurr Jr, 1985). The organophosphates include chemicals such as thionazin, ethoprophos, fenamiphos, fensulfothion, terbuufos, tsazofos, ebufos while the carbamates include aldicarb, aldoxycarb, oxamyl, carbofuran and cloethocarb (Spurr Jr, 1985; Giannakou et al., 2002; Abdel-Rahman et al., 2008; Onkendi et al., 2014; Jones, 2017). Macrocyclic lactones (avermectins and milbemycins) are other forms of non-fumigants which are chemical derivatives of soil microorganisms

belonging to the genus *Streptomyces* (Jayakumar, 2009 cited by Khalil, 2013). The avermectins include the group of ivermectin, abamectin, doramectin, eprinomectin, and selamectin which is a family of 16-membered macrocyclic lactones. Abamectin is a potent anthelmintic, insecticide, and miticide used to control pests of humans, animals and crops (Soyuncu et al., 2007).

The use of chemical control is economically viable for only high value crops (Tsay et al., 2004). Chemical control in agriculture increases production, however there are drawbacks associated with their application, problems with toxicity to animals and humans, poor target specificity and they are also not cost-effective, especially to resource poor farmers (McSorley et al., 2008; Collange et al., 2011; Ogumo, 2014; Youssef and Eissa, 2014; Danahap and Wonang, 2016). The incorrect use of chemicals, such as overdose, frequent applications, and application of pesticides beyond expiry date as well as illiteracy of applicators is common in developing and less developed countries (Gahukar, 2014) and this has a negative impact on crop production. Anju et al. (2010) stated that when nematicides, fertilizers, herbicides, insecticides and fungicides are applied to cropland, some residues remain in the soil after plant uptake and they may leach into the subsurface waters or they may move to surface water by dissolving in runoff or adsorbing sediment. These residues transform into products that may also contaminate water due to chemical and physical processes and this poses significant risks to the environment and non-target organisms ranging from beneficial soil microorganisms to insects, plants, fish and birds. As reported by Wallace (1973) cited in Olaniyi (2015), on low value and perennial crops, nematicides have been insufficiently effective, too expensive, phytotoxic or they may leave undesirable residues when applied on the growing crops. Pollution of the ozone layer

causing atmospheric ozone depletion can also occur (Soler et al., 2016) due to chemical pesticides. Moreover, their continued use can lead to some level of resistance in PPN species (Onkendi et al., 2014).

The majority of nematicides have been banned due to their adverse environmental effects. Methyl bromide was previously used as a multipurpose pre-plant broad-spectrum soil fumigant to control soil-borne diseases, nematodes, insects and weeds in high-value crops such as tomato, strawberry, cucurbits, nursery crops and flowers (Santos et al., 2006). It was known to provide excellent reduction of soil nematode populations, but its use was largely discontinued in the world after 2005 due to its harmful effects on human beings and to the environment, including beneficial organisms (Ibrahim et al., 2006; Renčo et al., 2014). Methyl bromide amongst others also has negative effects on other beneficial organisms, eliminating mycorrhizae which results in poorer plant growth (TNAU, 2015). The World Health Organization estimated over 370 000 deaths each year because of deliberate ingestion of pesticides (WHO, 2019).

2.2.1.1 Nematicide mode of action

The mode of action for organophosphates and carbamates is through inhibition of the acetylcholinesterase (AChE) enzyme **(Table 2.2)**. AChE degrades acetylcholine in the synapse (synaptic vesicle); thus, the inhibition of this enzyme allows accumulation of acetylcholine with subsequent excessive stimulation of acetylcholine receptors in associated postsynaptic cells and/ or end organs. The excessive inhibition of AChE (>50-60%) produces signs of toxicity which include autonomic dysfunction, for example excessive secretions of the salivary glands, muscle fasciculation and

respiratory depression (Pope, 1999). For example, fenamiphos as reported by Spurr Jr (1985) is a non-fumigant of the organophosphate class which interferes with normal nerve impulse transmission within the central nervous system of insects. Aldicarb is a form of carbamate that inhibits the infective nematode life stages from invading the root system where the chemicals are quickly taken up by the nematode and go through metabolism (Hartwig and Sikora, 1991). The mode of action of avermectins is through blocking the transmittance of electrical activity in nerves and muscle cells, by stimulating the release and binding of gamma-amino butyric acid (GABA) at nerve endings (Campbell et al., 1983; Burkhart, 2000) and disturbing neuromuscular transmission, leading to death (Martin et al., 2002).

Table 2.2: The mode of action of fumigants and non-fumigants used against nematodes

Nematicide group		Nematicides	Mode of action	Reference
Fumigants	Air (required for movement in soil)	Methyl bromide	Alkyl halides may react with nucleophilic sites on proteins and oxidize iron porphyrins and hemeproteins	Spurr Jr (1985)
		1,3 dichloropropene		
		Nemagon		
		Ethylene dibromide	Penetrates the body wall of the nematode (cuticle) directly	
		Chloropicrin		
	Water (required to activate toxicity in soil)	Metam-sodium	Penetrates the body wall of the nematode directly	Noling (1997)
		Dazomet	Affects internal organs	Spurr Jr (1985)
		Methyl isothiocyanate (MIT)	MIT may react with amino acids, oxidases and nucleophilic sites on proteins	
Nonfumigant	Organophosphate	Thionazin	Acts as narcotics, reduces fecundity	Kondrollochis et al. (1970); Reddy and Seshadri (1971)
		Ethoprophos	Acts as acetyl cholinesterase inhibitors	Noling (1997)
		Fenamiphos	Interferes with normal nerve impulse transmission within the central nervous system of insects	Spurr Jr (1985)
		Fensulfothion	Causes abnormal behaviour, paralysis and death.	
		Terbuufos	Penetrates the body wall of nematodes	
		Isazofos	Irreversible binding of acetylcholinesterase, esterase inhibition and various pharmacologic actions	
		Ebufos		
	Carbamates	Aldicarb	Inhibition of root invasion; chemical quickly taken up by the nematode and metabolised	Reddy and Seshadri (1971)
		Aldoxycarb	Penetrates the body wall of nematodes Act as acetyl cholinesterase inhibitors	Hartwig and Sikora (1991); Noling (1997)
		Oxamyl	Interferes with normal nerve impulse transmission within the central nervous system of insects	Spurr Jr (1985)
		Carbofuran	Causes abnormal behaviour, paralysis and death	
		Cloethocarb	Reversible binding of acetylcholinesterase, esterase inhibition and various pharmacologic actions	

2.2.2 Organic amendments

Organic amendments are known to have nematode suppressive effects which depend on many interactions that include the type of compounds released, the dosages, soil characteristics and the nematode population density (Collange et al., 2011). As reported by Akhtar and Malik (2000), plants and other organic matter used as soil amendments offer great benefits in terms of crop viability, soil nutrients, physical conditions, and soil biological activity. Through the decomposition process, organic matter residues stimulate the activities and increase abundance of microorganisms that are antagonistic to PPN (Akhtar and Malik, 2000). The most used organic amendments are wastes or by-products of agricultural industries, such as animal manures, compost and plant residues, grass and hedge clippings, shrubbery trimmings and tree parts (Akhtar and Malik, 2000; Oka, 2010). There are problems associated with using organic matter as control methods, such as inconsistent potency that is influenced by amendment and soil type (Oka, 2010), unavailability of materials, high transport costs, decreased soil pH which interfered with availability of some essential nutrient elements for plant growth and the large quantities required to achieve adequate suppression (Mashela, 2002). The main focus of the current study was on the use of plants as organic amendments and this is discussed below.

2.2.3 Phytonematicides

Natural plant products known as botanical pesticides or phytochemicals are the most studied organic amendments. They are known to be excellent candidates since they can be developed for use as nematicides themselves, or they can serve as model compounds for the development of chemically synthesized derivatives with enhanced activity or environmental friendliness (Chitwood, 2002). They are also known to be

compatible with other types of pesticides (Olaniyi, 2015). Application of plants to reduce the population densities of nematodes for crop protection can either be done by applying plants as soil amendments or using cover crops. Goswami and Vijayalakshmi (1986) conducted studies using pot cultures and *in vitro* studies for testing nematicidal properties of *Andrographis paniculata*, *Calendula officinalis*, *Enhydra fluctuans* and *Solanum khasianum* against *M. incognita*. All the plant materials reduced galls and nematode population densities in pot trials, with *C. officinalis* and *E. fluctuans* being most effective. Aqueous leaf extracts of *Strychnos nuxvomica* caused 100% mortality of J2 of *M. incognita* at a 2% concentration (Leela et al., 2012). Bawa et al. (2014) conducted a study on neem (*Azadirachta indica*), red-bell pepper (*Capsicum annuum*), ginger (*Zingiber officinale*) and African locust bean (*Parkia biglobosa*) which completely (100%) prevented attack and hatching of *M. incognita* J2 and also killed 100% of the latter life stage at 1 000 ppm concentrations (at 10% and above). Natarajan et al. (2006) investigated the effect of the cold-water extract of marigold (*Tagetes erecta*) on soil infested with *M. incognita* and reported that the plant effectively reduced root gall indices of tomato. Gall formation and multiplication of both *M. incognita* and *M. hapla* on tomato roots were significantly reduced by all soil treatments with annual wormwood (*Artemisia annua*) meal powder and water extract (D'Addabbo et al., 2017). Youssef and Lashein (2013) reported that amending the soil with crushed leaves of cabbage (*Brassica oleracea*) reduced the *M. incognita* population densities.

2.3 Method used for testing efficacy

Methods used in controlling nematodes such as chemicals, manures, phytonematicides or as cover crops exhibit one or more modes of action. The *in vitro*

and *in vivo* experiments are the most important methods in testing efficacy. *In vitro* testing includes J2 hatch, J2 mobility and J2 mortality. The *in vitro* activity should be confirmed with *in vivo* glasshouse pot trials, micro-plots and field trial experiments. A glasshouse is a controlled environment where temperature is maintained, and trials undertaken in these environments can give an indication of activity *in vivo*. Thereafter it is necessary to confirm the activity under natural conditions such as microplot and field trials where temperature and other environmental conditions are not controlled.

Ground leaching technology (GLT) is a method that was developed for use in smallholder farming to mitigate the drawbacks of conventional organic amendments in nematode suppression (Mashela, 2002; Mashela and Nthangeni, 2002; Mashela et al., 2017). The GLT method requires little plant material and involves spreading 5 g ground organic amendment per 15 cm radius in a shallow hole around the base of the stem at transplanting. The 5 g quantity amounts to 20 kg. ha^{-1} for 4 000 tomato plants/ha (Mashela and Nthangeni, 2002). In this technology, the active ingredients are leached out of the powdered plant material through irrigation water into the rhizosphere of plants with consistent effects on nematode suppression and fertiliser effects on tomato (Mashela, 2002; Mashela et al., 2017).

2.4 Animal-parasitic nematodes

Trichostrongylus colubriformis and *Haemonchus contortus* remain the most common nematodes that cause great loss of production in ruminants (Horak and Purnell, 2004). *Trichostrongylus colubriformis* causes parasitic enteritis that predisposes sheep to diarrhoea, weakness and death (Horak, 2003; Horak and Purnell, 2004). *Haemonchus contortus* causes haemonchosis in small ruminants and remains one of the most

important nematode parasites due to its high prevalence and pathogenicity (Hounzangbe-Adote et al., 2005). The loss due to haemonchosis in the livestock industry has been estimated to be tens of billions of dollars per annum (Waller and Chandrawathani, 2005; Roeber et al., 2013). The high fecundity of parasites, combined with the high rainfall and temperatures in the tropics favour permanent larval development throughout the year (Maphosa et al., 2010). According to Tariq (2018), the emergence and re-emergence of helminth disease in sheep is further worsened by changes in climate due to global warming.

Haemonchus contortus sucks blood, causing blood plasma and protein loss to the host as well as severe anaemia (Schoenian, 2003). The clinical signs of haemonchosis are usually non-specific and include various combinations of anorexia, anaemia, hypoproteinaemia, maldigestion, diarrhoea, weight loss, secondary submandibular oedema and ascites from the hypoproteinaemia and reproductive failure from poor condition (Soulsby, 1986; Taylor et al., 2007) or even death in severely infected animals (Burke et al., 2007).

2.4.1 Treatment options for animal-parasitic nematodes

Control strategies for animal-parasitic nematodes are based upon a combination of chemotherapeutic control, grazing management, dietary management, biological control, vaccination and ethnoveterinary medicine (EVM) treatment (FAO, 2002). Using rotational grazing, for example, by allowing cattle to graze for short periods will allow utilization of pastures while the *H. contortus* larvae population diminishes (Besier et al., 2016). However, this approach does not effectively remove the helminth burden compared to anthelmintic drugs (Krecek and Waller, 2006). The use of anthelmintic

drugs is restricted in animal use due to drug residues in animal products, environmental pollution and the development of anthelmintic resistance (Tariq, 2018).

2.4.2 Plant use as anthelmintics

Several indigenous plants have been identified as anthelmintics to manage gastrointestinal infections in small ruminants (Waterman et al., 2010; Simon et al. 2012; Adamu et al., 2013). In a study conducted by Eguale et al. (2011), the aqueous and hydro-alcoholic extracts of *Leucas martinicensis*, *Leonotis ocymifolia* and aqueous extract of *Senna occidentalis* and *Albizia schimperiana* induced complete inhibition of larvae from hatching at a concentration less than or equal to 1 mg/mL. The leaf and fruit aqueous and hydro-alcoholic extracts of *Maesa lanceolata* and aerial parts of *Plectranthus punctatus* completely inhibited larvae from hatching at a concentration of 1 mg/mL or below (Tadesse et al., 2009). Ethanol extracts of 25 plant species were screened for anthelmintic effects against *H. contortus* where five plants were reported to have high efficacy, namely *Ananas comosus, Lespedeza cuneata, Aloe ferox, Allium sativum* and *Warburgia salutaris.* The extracts of these plants had the highest larval motility varying from 68.0-96.1% at tested concentrations (10, 20 and 30%) (Ahmed et al., 2013).

Maphosa et al. (2010) tested the crude aqueous extracts of leaves of *Aloe ferox* and *Leonotis leonurus* and roots of *Elephantorrhiza elephantina* against the nematode parasite *H. contortus* for anthelmintic activities *in vitro* on the egg and larvae. *Elephantorrhiza elephantina* and *L. leonurus* extracts caused 100% egg hatch inhibition at 2.5 and 1.25 mg/ml respectively against *H. contortus* and caused total inhibition of larval development. Neem (*Azadirachta indica*) is also recommended for

use against gastrointestinal nematodes and related problems in many parts of the world (Subapriya and Nagini, 2005). Preliminary studies have shown that feeding foliage neem leaves is safe, eco-friendly, cheap and palatable to sheep (Sawleha et al., 2010). Feeding fresh leaves to animals reduced 82% of worm eggs (Chandrawathani et al., 2000). Costa et al. (2008) conducted the *in vitro* test using ethyl acetate and ethanol extracts of *A. indica* on *H. contortus* eggs and larvae. Ethanol extracts were effective in inhibiting the larval development by 87.11% at 50 mg/mL and inhibited egg hatch by 99.77% at 3.12 mg/mL. *Cymbopogon citratus* from the family Poaceae was reported in an ethnoveterinary study as having anthelmintic activity (Ritter et al., 2012). Citral, the major constituent of *C. citratus* essential oil, was found to be effective against *H. contortus* eggs and larvae (Macedo et al., 2015).

2.5 Is *Caenorhabditis elegans* a suitable test organism?

Caenorhabditis elegans is a free-living nematode belonging to the order Rhabditida, and is naturally found in temperate climate soils (Blaxter et al., 1998). As stated by Katiki et al. (2011), experimentation with *C*. *elegans* began in 1960 when researchers were looking for a multicellular organism with a few cells, easy to raise and reproduce for embryonic developmental studies. It was first used in 1965 to study animal development and behaviour by Sydney Brenner (Riddle et al., 1997) and in 1981 was used to screen potential anthelmintic compounds (Simpkin and Coles, 1981). Since then, *C. elegans* has become one of the most studied nematodes in many areas of biology. To work with parasitic nematodes is very difficult since they require a passage through their host for maintenance of their parasitic life-cycle (Holden-Dye and Walker, 2014). *Caenorhabditis elegans* is regarded as the most suitable test organism for preliminary high-throughput *in vitro* screening for compounds with broad-spectrum

nematicidal activity (Geary and Thompson, 2001). If tested drugs are effective against *C. elegans* cultures at low concentrations, it is reasonable to assume that they may have anthelmintic activity against related nematodes (Thompson et al., 1996). It provides the advantages of a rapid, low-cost *in vitro* laboratory method combined with the ability to examine activity of compounds against adult parasitic stages in related nematode species (Katiki et al., 2011).

The mode of action of anthelmintic drugs can be evaluated *in vitro* through nematode behaviour, locomotion and reproduction (Katiki et al., 2011). *Caenorhabditis elegans* has been reported to play a major role in defining the mode of action for nematicides as well as contributing to understanding mechanisms of resistance (Holden-Dye and Walker, 2014). In the development of vaccines against parasitic nematodes, *C. elegans* can be used as a model for the expression of vaccine antigens (Murray et al., 2007; Knox, 2012). However, in some reports, a poor correlation has been reported with activity against other phytoparasitic nematodes and high levels of broad-spectrum toxins have been identified instead of phytoparasitic nematode-specific compounds (Chitwood, 2002). Fluensulfone is a nematicide of the fluoroalkenyl thioether group and its mode of action was tested using *C. elegans*. The reported results showed that the effective dose required was higher than that to produce nematicidal activity on *Meloidogyne* spp. but the profile effects of *C. elegans* on motility, larval hatching and survival were similar to those reported for PPN (Kearn et al., 2014). In searching for nematicidal drugs, two or more test organisms should be used for screening, as broad-spectrum efficacy is of more value in the search for new compounds/drugs. This approach assists in avoiding the limitations that can be brought about by using single organisms (Aremu et al., 2012). It is therefore recommended to use this organism as

a test organism in bioassay guided fractionation of plant extracts for isolation of compounds and for studying mode of action of drugs on nematodes.

2.6 Plant selection

The selection of plants for pharmacological screening or drug discovery is very important and it involves five systematic approaches; namely ethnopharmacology, taxonomy, chemotaxonomy, random selection and the information-managed approach (Cordell et al., 1993). As reported by Brunht and Holmested (1981) cited in Karou et al. (2007), ethnopharmacology is the investigation of biologically active agents (plant mixtures, whole plants, a portion of a plant (leaf, root, stem), traditionally used or observed by humans. In the taxonomic approach, plant species belonging to families or genera considered to be of interest are sought from diverse locations while the chemotaxonomic approach is restricted to a certain class of secondary metabolites that have been identified as possessing activity (Colegate and Molyneux, 2007).

In the present study, the ethnopharmacological approach and targeted selection was used for the selection of plants. Plants were selected based on their known anthelmintic activity against free-living *C. elegans* and the animal-parasitic nematode *H. contortus*. The section below incorporates the description, distribution, traditional use, pharmacological and phytochemical information on the ten selected non-crop plant species.

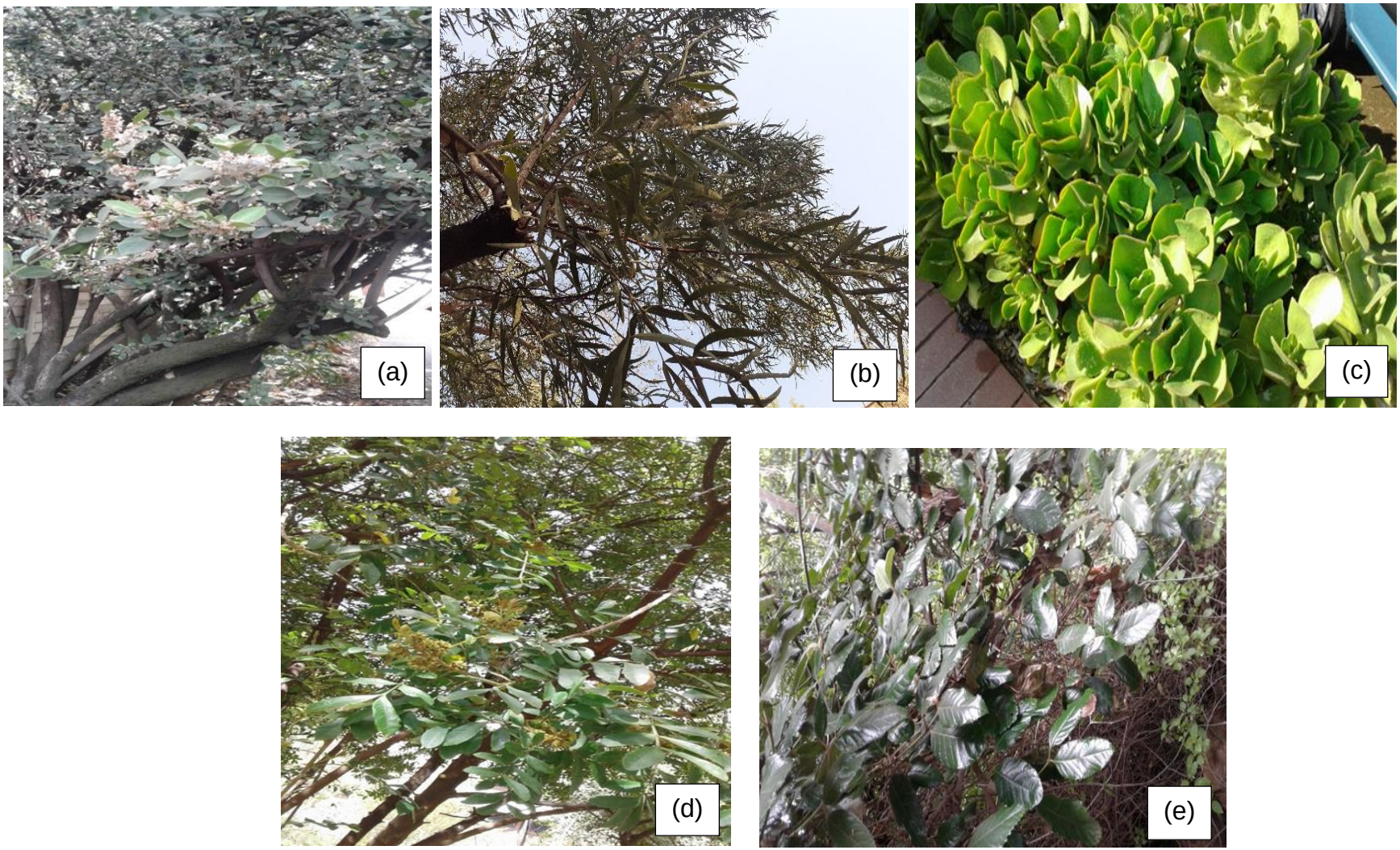


Figure 2.3: Plants selected for study: *Acokanthera oppositifolia* (a); *Searsia lancea* (b); *Cotyledon orbiculata* (c); *Hippobromus pauciflorus* (d) and *Curtisia dentata* (e) (Photos by FN Makhubu)

Figure 2.4: Plants selected for study: *Leucosidea sericea* (f); *Clerodendrum glabrum* (g); *Leonotis leonurus* (h) and (i) *Lantana rugosa* (Photos by FN Makhubu)

2.6.1 *Acokanthera oppositifolia* Lam. (Bushman's poison)

Acokanthera oppositifolia Lam. is a shrub or small tree belonging to the family Apocynaceae (van Wyk et al., 2009). It is widely distributed along the western and northern parts of South Africa (SA) and is also distributed in Mozambique, Zimbabwe, Zambia, Democratic Republic of the Congo, Malawi, Tanzania and Kenya (van Wyk et al., 2009). The tree has thick leathery leaves and red berries which turn dark purple

when ripe **(Figure 2.3a)**. All parts of the plant contain highly toxic latex (Watt and Breyer-Brandwijk, 1962a). The latex, fruit and decoctions of the wood of this plant are used as arrow poisoning in southern Africa (Adedapo et al., 2008) and in Swaziland, the root with cold water mixture of this plant is sprinkled around the house to prevent snakes from entering (Amusan, 2008).

The dried leaves and/or roots are used to treat headaches and snake bite while the extracts are used for colds, anthrax, and tapeworm infestation. According to the report of Iwalewa et al. (2007), leaves and roots of this plant are used for treating painful feet, rheumatism, toothache, abnormal menstrual periods and swelling. For application, the leaf or root pulp is rubbed into the wound while swollen parts are applied as a dressing (Watt and Breyer-Brandwijk, 1962). The powder made from the dry roots is used as a snuff for headache by Xhosa people in the Eastern Cape, SA and for snake bite where the paste made from crushed leaves and stem is applied to the site (Bhat and Jacobs, 1995). For the treatment of amenorrhoea, either leaves or roots of the plant are burnt and the smoke is directed into the vulva (Steenkamp, 2003). According to van Wyk et al. (2002), the plant is used for treating tapeworms, and people in Eastern Cape, SA as reported by Maphosa and Masika (2010) use a leaf decoction as an anthelmintic agent in goats.

In pharmacological reports, the plant has been found to have antimicrobial (Aremu et al., 2010; Mabona et al., 2013) anthelmintic (Aremu et al., 2010), antiplasmodial (Beourou et al., 2013), anti-inflammatory (Aremu et al., 2010; Ondua et al., 2016) and antioxidant activity (Adedapo et al., 2008; Amoo et al., 2012). The root extract was reported to have anti-cancer properties against panels of human cancer cell lines

(Fouche et al., 2016). Toxicity was reported from the roots and stems on a renal cell line (TK10), and moderate activity was found on breast MCF7 and melanoma UACC62 cells (Fouché et al., 2008). According to Kee et al. (2008), the aqueous stem and root extracts of *A. oppositifolia* (containing tannins) were found to have an effect on the $CaCl_2$ induced clotting time assay. Leaf and root extracts revealed the presence of condensed tannins, gallotannin, phenols and a small quantity of flavonoids (Aremu et al., 2010) whereas Amoo et al. (2012) quantified a smaller amount of phenolics, flavonoids, free gallic acid, gallotannins, while condensed tannins were not detected. Fresh roots contain a high quantity of iridoids. Acovenoside A and cardenolide (cardiac glycosides) are toxic compounds in *A. oppositifolia* and these are known to cause heart failure due to cardiac abnormalities (Steenkamp, 2003).

2.6.2 *Searsia lancea* (L.f.) F.A. Barkley (Karee tree)

Searsia lancea (L.f.) F.A. Barkley is a tree belonging to the Anacardiaceae family. The Anacardiaceae family (mango) belongs to the order Sapindales, which is represented by 77 genera and 600 species, mostly distributed in tropical, subtropical and temperate areas (Mabberley, 1997) genus *Rhus* (now known as *Searsia*) encompasses around 250 species of flowering plants (Tabassum et al., 2017). *Searsia* spp. comprise mostly small trees and shrubs, often with a resinous bark and milky sap; leaves are alternate, simple or compound, flowers are bisexual or unisexual (Kossah et al., 2010) **(Figure 2.3b)**. Species of the genus are found mostly in temperate and tropical regions worldwide (Rayne and Mazza, 2007). The fruits of various species of *Searsia* are considered to contain important ingredients, and roots are traditionally fermented (van Wyk and Gericke, 2000). According to Aganga and Mosase (2001), the seeds of *S.*

lancea are eaten by people and birds while the trees are used for fencing, thatching and the bark for tanning.

Searsia lancea has been reported to have good antimicrobial activity by several researchers. The aqueous and ethanolic stem bark extracts were reported to be active against *Staphylococcus aureus*, *Streptococcus pyogenes*, *Bacillus cereus*, *Salmonella typhi*, *Shigella sonnei* and *Escherichia coli* while leaf extracts were not active against all tested microbes (Obi et al., 2003). Hexane and methanol (bark and leaf) extracts were reported to have antibacterial activity against *Staphylococcus aureus* and anthelmintic activity against *C. elegans* (McGaw et al., 2007). Antimycobacterial activity was also reported in the acetone leaf extract (Mayekiso et al., 2009).

Compounds such as ∝-pinene, benzene, δ-3-carene, isopropyl toluene, and trans-caryophyllene, were found to contain medicinal properties such as antibacterial, antifungal and antioxidant activities (Gundidza et al., 2008). Nair et al. (1983) isolated two new flavonoids (7,4'-d-1-0-methylmyricetin and 3-0-galactoside) from the ethanol leaf extract along with three common flavonols - kaempferol, quercetin, and myricetin. The seeds were reported to have tannins and minerals such as phosphorus, calcium, potassium, magnesium, sodium, manganese, iron, copper and zinc (Aganga and Mosase, 2001).

2.6.3 *Cotyledon orbiculata* L. (pig's ear)

Cotyledon orbiculata L. is an herb belonging to the family Crassulaceae (van Wyk et al., 1997). The plant flowers during the spring and summer months and carries stout

erect branches with the leaves not clustered at the end **(Figure 2.3c)** (Terblanche and Adelaar, 1965). *Cotyledon orbiculata* is widely distributed in South Africa and commonly known as pig's ear. It is known locally as "Seredile" in Sotho and Tswana, "Plakkie" in Afrikaans and "Imphewula" in Xhosa (Watt and Breyer-Brandwijk, 1962b; Watt and Breyer-Brandwijk, 1962a; van Wyk et al., 1997). Traditionally, the plant is used for treating ear infections (Bhat and Jacobs, 1995). The name pig's ears is derived from the oval shape of the leaves (SANBI, 2019a). Xhosa people in Umtata, Eastern Cape, SA and Pedis in Limpopo Province, SA use the decoction of leaves for treating earache and inflammation. They also heat the leaf to treat swollen parts in the body and for removing warts; the juice in the leaves is applied on warts every morning for a week (Bhat and Jacobs, 1995; Wink and van Wyk, 2008; Maroyi and Mosina, 2014). The local communities of Thulamela Municipality in Limpopo Province, SA and Dr. JS Moroka in the Mpumalanga province, SA use the roots and leaves of *C. orbiculata* for treating sexually transmitted diseases and chronic diarrhoea (Masafu et al., 2016).

The plant is known to be toxic to livestock and causes serious chronic disease known as krimpsiekte, or “shrinking disease” (Terblanche and Adelaar, 1965; Wink and van Wyk, 2008). According to Wink and van Wyk (2008), meat from poisoned animals has been reported to cause secondary poisoning of dogs and humans. In the Cape, fresh leaf juice is used for treating epilepsy (Hutchings et al., 1996; Wink and van Wyk, 2008). Leaves are eaten as a vermifuge and applied as a hot poultice to treat boils, earache, and inflammation (Watt and Breyer-Brandwijk, 1962; van Wyk et al., 1997). The extracts of *C. orbiculata* have demonstrated great potential for anticonvulsant properties (Amabeoku et al., 2007). Antimicrobial activity has been reported in the

dichloromethane:methanol extract of *C. orbiculata* against gentamicin and methicillin resistant *Staphylococcus aureus* (MIC = 1.0 mg/mL), *Staphylococcus epidermidis* (MIC = 0.38 mg/mL), *Pseudomonas aeruginosa* (MIC = 0.50 mg/mL), *Candida albicans* (MIC = 0.25 mg/mL) and *Propionibacterium acnes* with MIC value of 0.25 mg/mL (Mabona et al., 2013). *In vitro* cultures and *ex vitro* plants were also reported to have antibacterial activity (Kumari et al., 2016).

The plant has been reported to be non-toxic to mice when testing for antinociceptive activity at the highest concentration of 400 mg/kg (Amabeoku and Kabatende, 2011) . It also has anti-inflammatory (Aremu et al., 2010; Amabeoku and Kabatende, 2011) and anthelmintic activity (Aremu et al., 2010). The leaf extracts were tested for their ability to inhibit *in vitro* the GABA-A-benzodiazepine receptor. Although they did not exhibit good activity, it was suggested that they may act via a different mechanism related to the traditional use (Stafford et al., 2005). The leaf extracts were also found to be good sources of possible antidepressant effects on the serotonin reuptake transport protein (Nielsen et al., 2004).

The phytochemical constituents in the leaves of *C. orbiculata* comprise cardiac glycosides, saponins, tannins, reducing sugars and triterpene steroids (Amabeoku et al., 2007) while flavonoids, condensed tannins, gallotannin and total phenolics were reported by Aremu et al. (2010). Iwalewa et al. (2007) reported tyledoside and bufanolide components. Orbicusides A, orbicusides B, orbicusides C and tyledoside C were also reported in *C. orbiculata* (Steyn et al., 1986).

2.6.4 *Hippobromus pauciflorus* (L.f.) Radlk. (False horsewood)

Hippobromus pauciflorus (L.f.) Radlk is a small tree **(Figure 2.3d)** belonging to the family Sapindaceae. It is used by traditional healers for the treatment of malaria (Clarkson et al., 2004). The leaf sap of this plant is used for managing inflamed eyes in animals and humans while the root and leaf infusions are used for clearing mucus from the noses of sheep and goats (Watt and Breyer-Brandwijk, 1962b; McGaw and Eloff, 2008). As documented by Masika et al. (2000), the leaf decoctions are used by small-scale livestock farmers in the Eastern Cape, SA for treating eye inflammation and Soyelu and Masika (2009) surveyed the use of the infused leaves as a wash for wounds in cattle in the Eastern Cape. The bark is used for treating heartwater and diarrhoea in cattle (Dold and Cocks, 2001; McGaw and Eloff, 2008). According to the ethnobotanical survey conducted by Wintola and Afolayan (2010) in the Nonkobe Municipality in Eastern Cape, the fresh root of *H. pauciflorus* is administered orally as a decoction for treating constipation.

According to Pendota et al. (2009), the plant possesses analgesic, antipyretic and anti-inflammatory effects. Antioxidant (Olorunnisola et al., 2012), antimicrobial (McGaw et al., 2007; Pendota et al., 2009) and anthelmintic activity (McGaw et al., 2007) were also reported. McGaw et al. (2007) reported safety of the extracts when testing against brine shrimp larvae, but Pendota et al. (2010) reported mild and dose-specific haemato-, hepato- and nephrotoxic effects of the leaf in male rats, which suggests that it is not completely safe as an oral remedy (Pendota et al., 2010).

Phytochemical analysis on this plant as reported by Olorunnisola et al. (2012) revealed the presence of flavonoids, flavonols, proanthocyanidins and tannins while Pendota et

al. (2009) reported tannins, flavonoids, steroids, terpenes, cardiac glycosides and saponins.

2.6.5 *Curtisia dentata* (Burm.f.) C.A.Sm. (Assegai tree)

Curtisia dentata (Burm.f.) C.A.Sm. is a tall evergreen tree **(Figure 2.3e)** belonging to the family Cornaceae. The flowers of this plant are small, drab cream-or fawn-coloured and velvety. The tree is not endemic to South Africa, and is widely distributed in the Eastern Cape, KwaZulu-Natal, Limpopo, Mpumalanga and Western Cape provinces of SA. It is listed as “Near threatened” under national status (Williams et al., 2008). It is commonly called assegai (English); assegaai (Afrikaans); umPhephelelangeni (Zulu) and modula-tshwene (Northern Sotho) (Williams et al., 2008). Medicinally, *C. dentata* is used as a blood enhancer, as an aphrodisiac, for the treatment of pimples, and as a treatment for heartwater in cattle in the Eastern Cape (Dold and Cocks, 2001; Shai et al., 2009). It is also used for treating sexually transmitted infections, stomach ailments, diarrhoea, heart-water, diabetes and obesity, and as a blood purifier and aphrodisiac (Cunningham, 1988; Hutchings et al., 1996). The leaves and the bark decoction are used by the people in the Eastern Cape for treating cancer (Koduru et al., 2007).

The leaves were found to have antifungal activity (Shai et al., 2008; Shai et al., 2009; Fadipe et al., 2015), while the stem bark was found to have antibacterial (McGaw et al., 2000; Shai et al., 2009; Doughari et al., 2012; Fadipe et al., 2015; Wintola and Afolayan, 2017), antimycobacterial (Fadipe, 2015) anthelmintic (Shai et al., 2009), antioxidant (Fadipe et al., 2015; Wintola and Afolayan, 2017; Olaokun et al., 2017), and anti-inflammatory activity (Olaokun et al., 2017). *Curtisia dentata* extracts have

been found to enhance glucose utilization of 3T3-L1 adipocytes which means the plant has potential to overcome insulin resistance in the cells and thus has antidiabetic potential (Olaokun et al., 2017). Shai et al. (2008) isolated four antibacterial and antifungal triterpenoids (lupeol, betulinic acid, ursolic acid, and 2α-hydroxyursolic acid) from the leaves of *C. dentata* and Fadipe (2015) tested these compounds for antibacterial and antifungal activity. The stem bark was found to contain high levels of flavonoids, total phenol and tannins, a low equivalent quantity of saponins and proanthocyanidin and very low alkaloids (Wintola and Afolayan, 2017). Similarly, saponins, tannins, glycosides, anthraquinones, flavonoids, steroids, and phenols were also detected (Doughari et al., 2012) while Olaokun et al. (2017) quantified polyphenols and flavonoids.

2.6.6 *Leucosidea sericea* Eckl. & Zeyh. (Oldwood)

Leucosidea sericea Eckl. & Zeyh. is a straggly shrub or a dense, small, evergreen tree **(Figure 2.4f)**, which grows up to 7 m tall and belongs to the Rosaceae family. In South Africa*, L. sericea* occurs in seven provinces, namely Eastern Cape, North West, KwaZulu-Natal, Free State, Gauteng, Mpumalanga and Limpopo of SA (van Wyk et al., 2008). The plant is commonly called oldwood (English), ouhout (Afrikaans), umtshitshi (Zulu) and cheche (Sesotho) (Foden and Potter, 2005c) Traditionally, it is used to expel parasitic intestinal worms, as an astringent with other plants, and it is also used as a treatment for conjunctivitis (Aremu et al., 2010).

The plant has been reported to have antimicrobial (Bosman et al., 2004; Aremu et al., 2010; Pitso and Ashafa, 2015), antibacterial (Sharma et al., 2014), antioxidant (Aremu et al., 2011), anthelmintic (Aremu et al., 2010; Adamu et al., 2013) and anti-

inflammatory activity (Aremu et al., 2010; Sharma et al., 2014). The extracts of this plant were found to have high inhibition potential of acetylcholinesterase and were not mutagenic (Aremu et al., 2011) nor cytotoxic to Vero monkey kidney cells (Adamu et al., 2013). The essential oil from *L. sericea* was not active against various microorganisms (Samie et al., 2012), but was found to inhibit the growth of *Fusarium oxysporum*, *Fusarium nygamai* and *Fusarium graminearum* with 10, 15 and 12 nm respectively when using the hole plate agar diffusion method (Samie and Nefefe, 2012).

Gallotannin, condensed tannin, flavonoids and total phenolics were identified in the leaves of *L. sericea* (Aremu et al., 2010). The cholestane triterpenoids β-sitosterol and β-sitostenone were isolated for the first time from the stems of *L. sericea* and had good anti-inflammatory activity (Nair et al., 2012). The phytol acetate, triacontanol, phytol and alpha kosin and one new compound, namely (E)-3,7,11,15-tetramethylheptadec-2-ene-1,17-diol (Sharma et al., 2014) and aspidinol and desaspidinol have been isolated from the leaves (Bosman et al., 2004). Phloroglucinol derivatives (agrimol A and G) and β-sitosterol were isolated by Adamu (2013). The extraction and isolation of essential oil from leaves of this plant and the subsequent GC-MS analysis yielded more than 50 compounds and β-pinene, limonene, isopinocarveol, p-menth-1-8-ol, and α-thujone were found to be the most dominant compounds (Pitso and Ashafa, 2015).

2.6.7 *Clausena anisata* (Willd.) Hook.f. ex Benth. (Horsewood)

Clausena anisata (Willd.) Hook.f. ex Benth. is a small tree **(Figure 2.4g)** belonging to the family Rutaceae. The plant is not endemic to South Africa, however its distribution

in the country covers most SA provinces including the Eastern Cape, Free State, KwaZulu-Natal, Limpopo, Mpumalanga and Western Cape (Foden and Potter, 2005a).

In African traditional medicine, the tree is reported to be used as a blood tonic, for treating epilepsy, malaria, taeniasis, schistomiasis, convulsions, influenza and other respiratory ailments, heart disorders and hypertension, abdominal cramps; constipation and gastroenteritis, hepatic diseases causing bad breath, malaria, fevers and pyrexia, boils, rheumatism, arthritis and other inflammatory conditions, headaches, body pains, toothaches and swollen gums, convulsions and some mental disorders, impotence and sterility, and dysentery in cattle (Hutchings et al., 1996; Ojewole, 2002). The decoction of the leaves is given as a stomachic and laxative after childbirth and it is also used for the treatment of some gastrointestinal disorders (Nukenine et al., 2010).

The plant has been reported to have several biological activities. It was an effective inhibitor of HIV-1 and HIV-2 replication (Ayisi and Nyadedzor, 2003), and had antidiabetic (Ojewole, 2002), anti-inflammatory (Okokon et al., 2012), anthelmintic (Adamu et al., 2013; Williams et al., 2016), anticonvulsant (Kenechukwu et al., 2012) antipyretic (Okokon et al., 2012) and antinociceptive activities (Okokon et al., 2012). It was also reported to have wound healing properties (Agyepong et al., 2015). Other important activity reported includes antibacterial (Senthilkumar and Venkatesalu, 2009; Lawal et al., 2015; Tankeo et al., 2015); antimicrobial (Agyepong et al., 2014; Makirita et al., 2016), antifungal (Hamza et al., 2006; Seshathri, 2012), and antioxidant (Agyepong et al., 2015; Lawal et al., 2015; Yakoob et al., 2016; Pavela et al., 2018). Risa et al. (2004) reported weak antiepileptic and anticonvulsant activity while

(Munodawafa et al., 2013) reported weak activity against *Candida albicans*. The leaf of *C. anisata* was found to have cytotoxic effects against brine shrimp larvae (Makirita et al., 2016), but Adamu et al. (2013) reported non-toxicity against Vero monkey kidney cells with LC_{50} value of 0.17186 mg/mL.

In pest management, the plant was reported to be effective as a mosquito repellent (Mukandiwa et al., 2016; Mavundza et al., 2018), as an antiplamosdial (Irungu et al., 2012; Okokon et al., 2012) and antifeedant (Pitan et al., 2009), and it also has insecticidal properties (Ndomo et al., 2008; Govindarajan, 2010; Nukenine et al., 2010; Pavela et al., 2018).

The phytochemistry of *C. anisata* has been extensively studied. It was reported to contain saponins, flavonoids, tannins and alkaloids (Kenechukwu et al., 2012), tannins, flavonoids, steroids, saponins, glycosides and alkaloids (Agyepong et al., 2014), flavonoids, triterpenes, phenols, saponins and alkaloids (Kuete et al., 2014), phenolics, alkaloids, flavonoids, saponins, proanthocyanidins and tannins (Lawal et al., 2015). As reported by Arsia et al. (2014) cited in Lawal et al. (2015), the leaves of *C. anisata* were found to contain alkaloids, flavonoids, carbohydrates, saponins, tannins, coumarins, proteins and amino acids. A total of eighteen compounds was detected in the essential oil and the major components were β-pinene, 1,8-cineole, pulegone, estragole and sabinene (Senthilkumar and Venkatesalu, 2009). Govindarajan (2010) also identified 18 compounds of the essential oil, with pinene, sabinene, germacrene-D, estragole and linalool being the major compounds. Also, Pavela et al. (2018) reported 58 chemical compounds, with phenylpropanoids (E)-anethole, (E)-methyl isoeugenol, and methyl chavicol being the major components

detected from the essential oil distilled from the leaves. Ngassoum et al. (1999) analyzed components in the essential oil from the leaf which were (2)-tagetenone, (El-tagetenone, (E)-nerolidol, and germacrene D while in the seed oil the major components were (2)-tagetenone, (E)-tagetenone, (El-nerolidol, myrcene, kcaryophyllene, 3-carene, and a-humulene, with some interesting minor compounds myrcenone (= ipsdienone), (E)-2(3)- and (Z)-2(3)-tagetenone epoxide and ambrettolide (mainly in the seed oil). Three carbazole alkaloids: girinimbine, murrayamine-A and ekeberginine; two peptide derivatives: aurantiamide acetate and N-benzoyl-L-phenylalaninyl-N-benzoyl-L-phenylalaninate; and a mixture of two phytosterols: sitosterol and stigmasterol were isolated from the stem bark and the roots of *C. anisata* (Songue et al., 2012) and were found to be the most dominant compounds. Two osthol compounds were isolated from the roots of *C. anisata*, 2H-1-benzopyran-2-one and 7-methoxy-8-(3-methyl-2-butenyl) (Pitan et al., 2009).

Four anisocoumarins were isolated from the leaves of *C. anisata*, namely anisocoumarins E, F, G and H (Ngadjui et al., 1989). The pyranocoumarin, seselin, was isolated from the leaf extracts of *C. anisata* and reported to have antifeedant activity (Mukandiwa et al., 2013; Mukandiwa et al., 2016). A total of eight known compounds, mukonal, glycosinine, mukonidine, clausine F, clausamine D, clausamine E, clausamine B, and clausamine C and two carbazole alkaloids named furanoclausamines A and furanoclausamines B have been isolated (Ito et al., 2009). Clausamine D, clausamine E, clausamine F, and clausamine G were isolated from the branches of *C. anisata* and were found to be good inhibitors of Epstein-Barr virus (Ito et al., 2000). The ethanolic stem extract of *C. anisata* was analysed using GC-MS and the plant was found to contain nine compounds with n-hexadecanoic acid, 8-

octadecenoic acid and methyl ester being major compounds (Yakoob et al., 2016). Clausenol (l-hydroxy-6-methoxy-3-methylcar- bazolc) and clausenine (1,6-dimethoxy-3-methyl carbazole) were isolated from alcoholic stem bark extract (Chakraborty et al., 1995).

2.6.8 *Clerodendrum glabrum* E. Mey. var. *glabrum* (tinderwood)

Clerodendrum glabrum E. Mey. var. *glabrum* is a shrub that grows to a height of just over a metre **(Figure 2.4h)** and belongs to the family Verbenaceae. It is widely distributed in the Northern Province of South Africa, North West, Gauteng, Mpumalanga, KwaZulu-Natal and Eastern Cape (Foden and Potter, 2005d). The roots and bulbs have been reported to be used for treating coughs and diarrhoea, and it has also been used to treat snake bites, intestinal parasites, coughs, fever and diabetes (Ndlovu et al., 2013; Adamu et al., 2014), and diarrhoea (Bisi-Johnson et al., 2010). The plant has been reported to be used to treat candidal infections by traditional practitioners in Venda (Masevhe et al., 2015).

Wahba et al. (2011) reported *C. glabrum* to have anti-inflammatory and anti-pyretic activity. Other pharmacological reports include antimicrobial (Suliman, 2010), antifungal (Masevhe et al., 2013), antibacterial (Ngobeni, 2012; Dzoyem et al., 2016), anthelmintic (Adamu et al., 2013) and antioxidant activities (Ndlovu et al., 2013).

Six compounds were isolated from the leaves of *C. glabrum*, including butyl-β-D-fructofuranoside, fructopyranoside, n-butyl-α-D-fructofuranoside, O- (β -D-apiofuranosyl)-mussaenosidic acid, ferulic acid and 3,5 dimethoxy benzoic acid (Wanas et al., 2013). Clerodendrumic acid and a known compound, heptadecanoic

acid, were isolated for the first time from the leaves of *C. glabrum* (Masevhe et al., 2013).

2.6.9 *Leonotis leonurus* (L.) R. BR.) (wild dagga)

The Lamiaceae (mint family) is a cosmopolitan family with 7 136 species occurring in 236 genera. In South Africa, the Lamiaceae family has about 308 species in 41 genera (Hussein, 2018). The genus *Leonotis* (Pers.) R.Br. comprises about 10 species (Naidoo et al., 2011) and 7 of the species occur in South Africa (Hussein, 2018). *Leonotis leonurus* (L) R. BR. is a perennial shrub of about 2 to 5 meters with a thick woody base, pale brown branches, and bright orange, hairy and tubular flowers **(Figure 2.4i)** with a strong smell in all parts. The hairy flowers resemble lion's ears, hence the species name "*leonurus"* which means lion's ears (van Wyk and Gericke, 2000). It is commonly called wild dagga or lion's ear in English (van Wyk and Gericke, 2003), umunyane in isiZulu, lebake in Sesotho, umfincafincane in Xhosa and is widely distributed in South Africa (van Wyk et al., 2002). *L. leonurus* is native to South Africa (He et al., 2012).

In South African traditional medicine, the leaves and the roots are used for treating various ailments such as coughs, colds, boils, influenza, diabetes mellitus, menstruation disorders, eczema, chest infections, bronchitis, hypertension, headaches, constipation, hepatitis, asthma, snake bites, insect bites and stings from scorpions (Jäger et al., 1996; van Wyk and Gericke, 2000; van Wyk et al., 2002). The plant has been smoked to relieve epilepsy (van Wyk and Gericke, 2003) while the decoction is drunk for water retention, obesity and digestive tract problems, intestinal worms and constipation (SANBI, 2018). The tea has also been used effectively for the

treatment of jaundice, cardiac problems, asthma, haemorrhoids, headaches, chest aliments, bronchitis and epilepsy (SANBI, 2018). A survey conducted in the Eastern Cape revealed that *L. leonurus* leaves are used by farmers to treat helminthosis in goats (Maphosa and Masika, 2010).

According to Steenkamp et al. (2004), phenolic compounds from the Lamiaceae family allow the plants to have a diverse range of biological activity. The plant has been reported to have antidiabetic (Ojewole, 2005; Oyedemi and Afolayan, 2011), anti-inflammatory (Ojewole, 2005; Stafford et al., 2005; El-Ansari et al. 2009; Maphosa et al., 2012), antimicrobial (Hurinanthan, 2009), antibacterial (Kamatou et al., 2006; Jimoh et al., 2010; Madureira et al., 2012), antifungal (Motsei et al., 2003), antioxidant (Hurinanthan, 2009; Jimoh et al., 2010; Oyedemi and Afolayan, 2011), anthelmintic (Maphosa et al., 2010), analgesic (Maphosa et al., 2012), antiplasmodial (Hurinanthan, 2009), antinociceptive (Ojewole, 2005) and anticonvulsant activity (Bienvenu et al., 2002). In the report of Bienvenu and colleagues, anticonvulsant effect was via non-specific mechanisms when tested against seizures produced in mice by pentylenetetrazole, picrotoxin, bicuculline and N-methyl-DL-aspartic acid (intraperitoneal injections). It was found to delay the latency of seizures produced by agents affecting both gabaergic and glutaminergic systems (Bienvenu et al., 2002). Risa et al. (2004) investigated the anticonvulsant activity of *L. leonurus* and found it to be inactive suggesting that the mechanism of action was not through the GABA-A-benzodiazepine receptor activity tested. The plant was reported to be a good inhibitor of phosphodiesterase (Mulubwe, 2007) and it has hepatoprotective properties (El-Ansari et al., 2009). Weak anthelmintic activity against *Caenorhabditis elegans* was reported in the leaf extracts (McGaw et al., 2000). However, Maphosa and Masika

(2010) reported good anthelmintic activity against *Haemonchus contortus* where hatching of eggs and larval development were inhibited by 100% at 1.25 mg/mL concentration.

Leonotis leonurus displayed no toxicity to human cell lines and no mutagenicity was reported at the highest concentration of 1 000 µg/mL (Hurinanthan, 2009). Again, no toxicity was shown by the plant against brine shrimps at the highest concentration of 1 000 µg/mL (Hurinanthan, 2009). However, in some reports the aqueous extract of *L. leonurus* displayed cytotoxic effects against HL-60 cells, an acute promyelocytic leukaemia cell line (Kee et al., 2008) and it was toxic in terms of hematological parameters and negative effects on various organs tested in rats which can lead to death (Maphosa et al., 2008).

Marrubiin, a constituent of *L. leonorus* was found to have antidiabetic, anti-inflammatory (Mnonopi et al., 2012), anticoagulant, antiplatelet and anti-inflammatory effects (Mnonopi et al., 2011). Cardioactive compounds from the plant were found to have specific cardiovascular activity *in vivo* (Obikeze, 2004). The essential oils reported from *L. leonurus* leaf and flower showed diverse phytochemicals such as limonene, (Z)-β-ocimene, terpinene, caryophyllene, humulene and germacrene (Steenkamp et al., 2004). The phytochemical analysis reported by Muhizi (2002) revealed the presence of alkaloids, tannins, terpenoids and quinones. Flavonoids, terpenoids and phenolics were reported in the study of Madureira et al. (2012) while phenolics, flavonoids, proanthocyanidins and flavonols were quantified by Oyedemi and Afolayan (2011). *Leonotis leonorus* was reported to contain an appreciable number of proteins, fat, fiber, carbohydrate and calorific value, mineral elements,

polyphenols and low levels of toxicants (Jimoh et al., 2010). Two novel labdane-type diterpenoids were isolated from the leaves of *L. leonurus* as 9,13-epoxy-6-hydroxy-labdan-16,15-olide and 9,13,15,16-diepoxy-6,16-labdanediol (Naidoo et al., 2011). A new diterpene ester, 1,2,3- trihydroxy-3,7,11,15-tetramethylhexadecan-1-yl-palmitate was isolated along with five known metabolites that are succinic acid, uracil, luteolin 7-O-glucoside, acteoside and geniposidic acid (Agnihotri et al., 2009), labdanes, leonurenones A-C, two known labdanes, luteolin 7-O-β-glucoside and luteolin (He et al., 2012), three known leoleorins (A-C) and eight hitherto unknown leoleorins (D-J and 16-epi-leoleorin F) and labdane diterpenoids (Wu et al. 2013), stachydrine was quantified in the aerial parts of *L. leonurus* (Kuchta et al., 2013). A novel dilactone diterpene ((13S)-9α, 13α-epoxylabda-6β (19),15(14) diol dilactone), was isolated from the leaves of *L. leonurus* and found to cause significant changes in blood pressure of anaesthetized normotensive rats and exhibited a negative chronotropic effect (Obikeze et al., 2008). Ten flavonoid compounds were isolated and identified as one diglycoside flavone, apigenin 6-C-α-arabinoside-8-C-β-glucoside; four monoglycoside flavones, apigenin 8-C-β-glucoside, apigenin 7-O-β-glucoside, luteolin 7-O-β-glucoside, and luteolin 7-O-β-glucoside-3'-methyl ether; one acylated monoglycoside flavone, apigenin 7-O- (6''-O-p- coumaroyl)-β-glucoside; two methylated flavones, 6-methoxyluteolin-4'-methyl ether and luteolin 3'-methyl ether; and two flavone aglycons, luteolin and apigenin (El-Ansari et al., 2009).

2.6.10 *Lantana rugosa* Thunb. (bird's brandy)

Lantana rugosa is a small shrub with lanceolate leaves **(Figure 2.4j)** belonging to the family Verbenaceae. This family is made up of 35 genera and over 1200 species with the genus *Lantana* comprising more than 150 species (SANBI, 2019b). It is commonly

called bird's brandy (English), mabele-mabutswapele (Sesotho) and utywala bentaka (IsiXhosa). The plant is widely distributed in all nine provinces of South Africa (Foden and Potter, 2005b). Traditionally, the plant is used for treating abdominal and chest complaints in children, sore eyes, bad sores, coughs, sprains and rheumatism (Stoffersen et al., 2011). The paste of this plant is applied to treat festering sores (Smith, 1895), coughs and chest complaints (Hutchings et al., 1996). Traditional healers of the Lwamondo area in Limpopo Province boil the roots and drink the mixture for fever (Mahwasane et al., 2013).

The plant has been reported to have antimicrobial (Mabona et al., 2013), antibacterial (McGaw and Eloff, 2005; Suliman, 2010) and anthelmintic activity (McGaw and Eloff, 2005). Cytotoxic activity of the plant was reported on brine shrimps with LC_{50} of 0.69 mg/mL (McGaw and Eloff, 2005). As reported by Watt and Breyer-Brandwijk (1962) and Hutchings et al. (1996) cited in McGaw et al. (2008), volatile oil and the alkaloid lantanin, as well as pentacyclic triterpenoids have been reported in *L. rugosa*.

CHAPTER 3

Comparative anthelmintic activity against free-living and animal-parasitic nematodes and phytochemical profiling of selected South African non-crop plant species

3.1 Introduction

Parasitic nematodes of ruminants are normally used as test systems for nematode control (Spiegler et al., 2017). However screening for anthelmintic drug development in animal models is often not ideal as experimenting in natural hosts is very expensive and appropriate facilities are required, in addition to concerns regarding animal welfare (Kumarasingha et al., 2014). *Caenorhabditis elegans* is a free-living nematode belonging to the order Rhabditida which is naturally found in temperate climate soils (Katiki et al., 2011). The order Rhabditida is closely associated with the order Strongylida, the group containing the important trichostrongyloid parasites of ruminants, which include species of *Haemonchus, Ostertagia, Trichostrongylus, Cooperia* and *Dictyocaulus* (Geary and Thompson, 2001). *Caenorhabditis elegans* is widely used as a model for anthelmintic drug development since it provides the advantage of low costs of an *in vitro* laboratory method with a life cycle of 3-5 days at 20˚C (Stiernagle, 2006; Kumarasingha et al., 2014).

Haemonchus contortus, a gastrointestinal strongyle species, is one of the most important and prevalent parasites causing haemonchosis in small ruminants (Hounzangbe-Adote et al., 2005). It is highly pathogenic with non-specific clinical signs of various combinations of weight loss, anaemia, diarrhoea, maldigestion and

reproductive failure from poor conditions (Soulsby, 1986; Taylor et al., 2007). It may cause death in severely infected animals (Burke et al., 2007). The high fecundity of parasites, combined with the high rainfall and temperatures in the tropics favours permanent larval development throughout the year (Maphosa et al., 2010). Losses in the livestock industry are estimated at US$10 billion per annum (Waller and Chandrawathani, 2005; Roeber et al., 2013).

Commercial anthelmintics have been used for decades throughout the world to minimize losses caused by helminth infections (Eguale et al., 2011). Excessive use of such drugs has led to widespread resistance in these nematodes to most classes of anthelmintics (Kaplan and Vidyashankar, 2012) and the problem of resistance is increasing worldwide (Geurden et al., 2015). Anthelmintic drugs can be harmful to humans in terms of food residues and they can also cause environmental pollution (Herd, 1996; Cooper et al., 2015). The high costs of such drugs also present restrictions to their use (Hounzangbe-Adote et al., 2005).

Traditional methods of controlling nematodes used by small farmers remain largely dependent on medicinal plants (Hounzangbe-Adote et al., 2005). Several studies have been conducted in terms of screening plants for use as anthelmintic agents using *C. elegans* as a model. In the development of anthelmintic drugs, it is important to note that when drugs are effective at low concentrations against *C. elegans* they might also be active against related nematodes, including *H. contortus* (Thompson et al., 1996). In research published by McGaw et al. (2007), seventy plant extracts (aqueous, methanol, and hexane) from 24 plants used in South African ethnobotanical medicine were evaluated to determine their possible anthelmintic activity on *C. elegans*. Twenty-

five extracts were found to be active with the hexane extract of *Hippobromus pauciflorus* demonstrating the highest activity, killing 70% of nematodes at a concentration of 0.5 mg/mL and 50% of nematodes at 2 mg/mL. Waterman et al. (2010) investigated the anthelmintic activity of the aqueous and organic extracts of 33 plant parts from 17 plant species used in traditional sub-Saharan African medicine. Of the 17 species tested, 12 plants showed significant activity against *C. elegans*.

Concerns have been raised about the toxicity and side effects of medicinal plants in long term use. According to Saad et al. (2006), toxicity of medicinal plants may be related to the active compounds they contain; either singly or in mixture, interactions with other herbs and drugs, contaminants, adulterants, or even their inherent toxicity. A cytotoxicity test is one of the most important preliminary indicators of toxicity *in vitro* (Li et al., 2015). Testing plants for cytotoxicity *in vitro* allows identification and prioritisation of plant extracts useful for further biological evaluation. It is therefore important to evaluate the safety of the active plant species under study to provide a measure for their usage as potential candidates for development of both anthelmintic and nematicidal product development.

The 3-(4, 5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium (MTT) assay is a rapid colorimetric test that was designed to measure only living cells (Mosmann, 1983). It is the most used method to test cell growth rate and toxicity of the culture (Li et al., 2015). MTT is known to be more accurate than other detection methods (Li et al., 2015). It is fast, user-friendly and the method is highly reproducible (Hatok et al., 2009). Many salts are used to detect viability of cells, however, MTT is the most commonly used

salt. Tetrazolium salts are reduced to a coloured formazan only by metabolically active cells, so the assay detects viable cells exclusively (Hatok et al., 2009).

Plants are natural resources used medicinally in different countries and they are a major source of many potent and powerful drugs (Dubal et al., 2013). Secondary metabolites constitute a large and varied group of organic compounds that are synthesized in small quantities in plants. They appear in plants as a result of chemical conversions and function to defend against predators and pathogens, to act as allelopathic agents and to attract seed pollinators (Mera et al., 2019). Profiling chemicals in plants is very important in product development, and understanding the constituents present in the plants aids in avoiding consumption of toxic plants. Gas chromatography-mass spectroscopy (GC-MS) represents one of the oldest and most successful hyphenations of separation techniques to a mass spectrometer (Wiles, 2011). It is the most common application for characterization of natural products for their chemical composition to aid antimicrobial activity studies, toxicological analyses, chemical ecological studies, the characterization of materials, and pharmaceutical/drug monitoring and clinical analyses to mention some (Sichilongo et al., 2012).

The current chapter aimed to confirm the anthelmintic activity of the previously reported plants against *C. elegans* and *H. contortus* using a range of extracting solvents, namely water, acetone, and dichloromethane: methanol (1:1). The *in vitro* safety of the active plants was also determined. The active plant extracts were further fractionated and tested for motility against *C. elegans* juveniles and inhibition of larvae

(L1)from hatching against *H. contortus*. Cytotoxicity of the active extracts and fractions was also evaluated.

3.2 Materials and Methods

3.2.1 Plant collection and extraction

3.2.1.1 Collection and identification

The fresh leaves from 10 plants were collected at the Manie van der Schijff Botanical Garden and experimental farm (University of Pretoria) as well as the Faculty of Veterinary Science grounds (University of Pretoria) in the spring months. Voucher specimens were prepared and deposited in the H.G.W.J. Schweickerdt Herbarium (PRU), University of Pretoria. The collected plants were authenticated by herbarium specialist, Ms Nel. Collected plants were *Leonotis leonurus* (L) R. BR (PRU 124359), *Clausena anisata* (Willd.) Hook.f. ex Benth (PRU 124385), *Hippobromus pauciflorus* (L.f.) Radlk (PRU 124360), *Leucosidea sericea* Eckl. & Zeyh (PRU 124358), *Lantana rugosa* Thunb (PRU 124386), *Clerodendrum glabrum* E. Mey (PRU 124362), *Curtisia dentata* (Burm.f.) C.A.Sm. (PRU 124361), *Acokanthera oppositifolia* (Lam.) Codd (PRU 124363), *Searsia lancea* (L.f.) F.A. Barkley (PRU 124364) and *Cotyledon orbiculata* L. (PRU 0125286).

3.2.1.2 Plant extraction

The collected leaf material was dried at room temperature in a well-ventilated room and ground to a fine powder in a Macsalab Mill (Model 2000 LAB Eriez). Succulent leaves of *C. orbiculata* were oven-dried at 45 to 50°C until a constant weight was reached. Twenty-five gram (25 g) of each plant was separately extracted in 250 mL of distilled water, acetone, and dichloromethane: methanol (1:1 v/v) (Minema Chemicals). The extraction was left at room temperature for 30 min and filtered

through Whatman No. 1 filter paper. The procedure was repeated twice to exhaustively extract plant material and left overnight (24 h) during the final extraction. All the organic filtrates were concentrated in a rotary evaporator and water extracts were dried in front of a stream of cold air. Extracts were stored at 8°C until use.

3.2.1.3 Fractionation

The powdered leaf material of *L. leonurus* (24 g), *C. anisata* (75 g), and *L. rugosa* (45 g) was extracted in 240 mL, 750 mL and 450 mL of acetone respectively. The extraction was left to proceed at room temperature for 30 min before the extract was filtered through Whatman No. 1 filter paper. The procedure was repeated twice to exhaustively extract plant material, left overnight 24 h for complete extraction, filtered and evaporated using a Büchi rotary evaporator (R-114, Labotec).

The acetone crude extracts of the three plants were dissolved in 20 mL of distilled water. The dissolved extract was reconstituted in 300 mL of hexane and 280 mL of distilled water was added to give equal volume. The *n*-hexane fraction was collected, and the water layer was mixed with an equal volume of dichloromethane to yield the dichloromethane fraction. The water layer was again mixed with an equal volume of ethyl acetate to yield the ethyl acetate fraction and the remaining water was portioned with *n*-butanol to give *n*-butanol and water fractions. The five collected fractions were evaporated and dried to result in *n*-hexane, dichloromethane, ethyl acetate, *n*-butanol and water fractions for the three plants.

3.3 Anthelmintic activity

3.3.1 Motility assay using *Caenorhabditis elegans*

Anthelmintic activity of plant extracts and fractions were assayed using the free-living nematode *C. elegans* var. Bristol (N2), which was cultured on nematode growth (NG) agar seeded with *Escherichia coli*, according to the method of Brenner (1974). Plant extracts and fractions were made up to 100 mg/mL concentration. Water, acetone and DCM/MeOH extracts were prepared in their respective solvents whereas fractions were re-dissolved in 10% DMSO. The samples were tested against juveniles at concentrations of 0.5, 1 and 2 mg/mL following the protocol of (Rasoanaivo and Ratsimamanga-Urverg, 1993), as modified by McGaw et al. (2000). About 100 nematodes (7 to 10-day-old cultures) in M9 buffer (Brenner, 1974) were incubated with different plant extracts for 24 and 48 h at 25°C in the dark. The anthelmintic drug levamisole (Sigma, 5 and 10 µg/mL) was used as a positive control. Using an inverted microscope, nematodes were counted and determined as motile or non-motile. Motility of hatched juveniles was defined as any unprovoked movement within a 5-sec interval (Skantar et al., 2005) and they were counted after 24 and 48 h. Nematodes were considered motile when they exhibited any movement from either the head or the tail, or pharyngeal movements and as non-motile when there was no tail, head or pharyngeal movements during 5 s of observation.

3.3.2 *Haemonchus contortus*

3.3.2.1 Collection and preparation of eggs

Ethical permission was obtained for this aspect of the study from the Animal Ethics Committee, University of Pretoria (V017-18). Three sheep were monospecifically infected with the infective larvae of *H. contortus*. They were kept indoors on a concrete

floor and fed with lucerne, teff, and commercial concentrate pellets while allowing free access to potable water. The temperature and weight of all animals were monitored and a FAMACHA chart was used by a veterinarian to check if the animals were anaemic. The nematode eggs were collected and prepared according to the method of the World Association for the Advancement of Veterinary Parasitology (WAAVP) with modifications (Coles et al., 1992). The faecal samples were mixed with water to make a relatively liquid suspension (slurry), which was serially filtered through sieves of sizes 250, 150, 90, 63, and 38 µm to trap the eggs on the latter. The material on the 38 µm mesh was washed into 50 mL centrifuge tubes, re-suspended in a magnesium sulphate solution (1:10), and centrifuged at 1000 x g for 10 min to separate the eggs from other debris. The resultant supernatant was passed through a 38 µm sieve to finally harvest the eggs into distilled water. The egg concentration was brought to a final concentration of 100 eggs with phosphate buffered saline (PBS) per 0.2 mL using a McMaster slide under a microscope.

3.3.2.2 Infective larvae (L1) hatch assay

The infective larvae hatch assay was conducted according to WAAVP guidelines (Coles et al., 1992). Egg suspension (0.2 mL) was distributed into wells of a 48-well flat-bottomed plate and mixed with 0.2 mL of each plant extract and fraction. The acetone and aqueous extracts were re-dissolved in their respective solvents while dichloromethane: methanol extracts and fractions were re-dissolved in 10% DMSO to give a concentration of 10 mg/mL. They were serially diluted to six concentrations,3.333 mg/mL, 1.666 mg/mL, 0.833 mg/mL, 0.416 mg/mL, 0.208 mg/mL and 0.104 mg/ mL. Albendazole (Sigma, Germany) (25 to 0.008 µg/mL dissolved in 5% dimethyl sulfoxide (DMSO) was used as the positive control and PBS buffer as the

negative control. After incubation at 27°C for 48 h, a drop of Lugol's iodine solution was added to each well to stop the eggs from hatching. The numbers of motile larvae (L1) and eggs were counted using an inverted compound microscope at 10× 10 magnification.

3.4 Cytotoxicity

3.4.1 MTT assay

The cytotoxic effect of the extracts and fractions on the viability of African green monkey kidney cells was investigated using the tetrazolium based colorimetric MTT assay described by Mosmann (1983). The cells were grown in Minimal Essential Medium (MEM) supplemented with 0.1% gentamicin (Virbac) and 5% foetal calf serum (Highveld Biological). Cells obtained from a subconfluent culture were centrifuged at 200 x g for 5 min and were resuspended in MEM growth medium to 5 x 10^4 cells/mL. Briefly, 200 µL of cell suspension was added to each well of columns 2 to 12 followed by the same volume of 200 µL MEM in the first column containing no cells (blank). The plates were incubated for 24 h at 37°C in a 5% CO_2 incubator to allow the cells to attach and reach the exponential phase of growth before treatment. The spent medium was removed and replaced with fresh MEM (100 µL) followed by the addition of 100 µL extract or fraction at various concentrations. The plates containing treatments and positive control (doxorubicin chloride, Pfizer Laboratories) were incubated for 48 h at 37°C in a 5% CO_2 incubator. Untreated cells were also included as negative control. The acetone and aqueous extracts were re-dissolved in their respective solvents while dichloromethane: methanol extracts and fractions were re-dissolved in 10% DMSO to give a concentration of 100 mg/mL. The 1 mg/mL extract and fraction obtained from the stock solution was serially diluted and prepared in serum-free MEM. Six

concentrations were used for the experiment, 0.1000 mg/mL, 0.0750 mg/mL, 0.0500 mg/mL, 0.0250 mg/mL, 0.010 mg/mL and 0.0075 mg/mL.

After incubation, the MEM from all the wells was removed and cells were washed with 200 µL of phosphate-buffered saline (PBS, Whitehead Scientific), followed by the addition of 200 µL of MEM and 30 µL MTT (Sigma, a stock solution of 5 mg/mL in PBS). The plates were further incubated for 4 h at 37°C. After incubation with MTT, the medium was removed and 50 µL of DMSO was added to each well and the plates were shaken gently to dissolve the MTT formazan crystals. Then the amount of MTT reduction was measured immediately by detecting absorbance in a microplate reader (BioTek Synergy) at a wavelength of 570 nm. The experiment was repeated three times and samples were tested in triplicate.

3.5 GC-MS Chemical profiling

The analyses using GC-MS were performed with the assistance of Dr. Yvette Naudé at the University of Pretoria, Department of Chemistry. GC-MS analysis was carried out using a LECO Pegasus 4D GC-TOFMS (LECO Africa (Pty) Ltd., Kempton Park, South Africa) with a Rxi-5SilMS GC column (30 m x 0.25 mm ID x 0.2 µm film thickness) (Restek, Bellefonte, PA, USA). The following parameters were used:

- Injection volume 1 µl, splitless injection, splitless time set at 30s
- GC inlet 250°C
- GC oven temperature programme 40°C (hold for 3 min) at 10°C/min to 300°C (hold for 5 min).
- Carrier gas used was UHP Helium (Afrox, South Africa) at 1 ml/min set at constant flow mode
- Mass acquisition range 40-550 Da

- MS transfer line temperature 280°C
- Ion source temperature 230°C
- MS solvent delay 5 min
- Electron energy 70 eV in the electron ionisation mode (EI+)
- Data acquisition rate 10 spectra/ s
- Detector voltage 1750 V.

The resulting data were combined and interpreted, and compounds corresponding to major peaks which showed high similarity (above 85%) were recorded.

3.6 Calculations and statistical analysis

All experiments were done in triplicate and repeated twice.

Percentage immotility for *C*. *elegans* was calculated using Abbott's formula (Abbott, 1925) as follows:

Immotility (%) = [(immotility percentage in treatment - immotility percentage in untreated control)/ (100-immotility percentage in untreated control)] ×100%.

The percent (%) inhibition of L1 on *H. contortus* was calculated using the formula modified after Coles et al. (1992):

Hatch inhibition (%) = 100(1-$P_{test}/P_{control}$); Where P_{test} *i*s the number of hatched L1 (Larval forms (L1)) and $P_{control}$ is the respective numbers in phosphate buffer (PBS).

Cytotoxicity

The $LC_{50\%}$ (concentration resulting in 50% reduction of absorbance) values were determined using linear regression model and presented as arithmetic mean values ±

standard error of mean. Percentage cell viability was calculated using the following formula:

Percentage cell viability (%) = [(mean absorbance of sample/mean absorbance of control)] X100%

3.7 Results and Discussion

The list of selected plant species with published anthelmintic activity against *H. contortus* and *C. elegans* is shown in **Table 3.1**.

Table 3.1: Medicinal plants screened for anthelmintic activity against *Haemonchus contortus* and *Caenorhabditis elegans* used in the present study

Plant species	Part used	Solvent used	Activity		Type of assay and organism tested	Reference
			CA (mg/mL)	DBA (mg/mL		
Acokanthera oppositifolia (Lam.) Codd.	Leaf	Petroleum ether	0.52	NT	MLC (*C. elegans*)	Aremu et al. (2010)
		Dichloromethane	1.04	NT		
		Ethanol	0.52	NT		
		Water	4.17	NT		
	Twig	Petroleum ether	2.08	NT		
		Dichloromethane	4.17	NT		
		Ethanol	2.08	NT		
		Water	2.08	NT		
Clausena anisata (Willd.) Hook.f. ex Benth.	Leaf	Hexane	NT	At 1 mg/mL: –and –	2 h and 7-day assay (*C. elegans*)	McGaw et al. (2000)
		Ethanol	NT	At 1 mg/mL: + and ++		
		Water	NT	At 1 mg/mL: + and +		
		Acetone	NT	2.07	LDA (*H. contortus*)	Adamu et al. (2013)
			NT	1.80	EHA (*H. contortus*)	
Clerodendrum glabrum E. Mey.	Leaf	Hexane	NT	At 1 mg/mL: – and –	2 h and 7-day assay (C. elegans)	McGaw et al. (2000)
		Ethanol	NT	At 1 mg/mL: -and –		
		Water	NT	At 1 mg/mL: -and –		
		Acetone	NT	12.97	LDA (*H. contortus*)	Adamu et al. (2013)
			NT	1.48	EHA (*H. contortus*)	

Table 3.1: Medicinal plants screened for anthelmintic activity against *Haemonchus contortus* and *Caenorhabditis elegans* used in the present study **Continued.**

Plant species	Part used	Solvent used	Activity		Type of assay and organism tested	Reference
			CA (mg/mL)	DBA (mg/mL)		
Cotyledon orbiculata L	Leaf	Petroleum ether	0.26	NT	MLC (*C. elegans*)	Aremu et al. (2010)
		Dichloromethane	1.04	NT		
		Ethanol	0.26	NT		
		Water	>16.67	NT		
	Stem	Petroleum ether	4.17	NT		
		Dichloromethane	4.17	NT		
		Ethanol	8.33	NT		
		Water	8.33	NT		
Curtisia dentata (Burm.f.) C.A.Sm.	Leaf	Acetone	NT	0.31	Larval mortality assay after 48 h (*H. contortus*)	Shai et al. (2009)
		Dichloromethane	NT	0.63	Larval mortality assay after 48 h (*H. contortus*)	
		Acetone	NT	0.31 to 0.63	7 days assay (*C. elegans*)	
		Dichloromethane	NT	0.31 to 0.63	7 days assay (*C. elegans*)	
Hippobromus pauciflorus (L.f.) Radlk	Arial part	Hexane	NT	At 0.5 mg/mL: 2	2 h assay (*C. elegans*)	McGaw et al. (2007)
		Water	NT	At 2 mg/mL: 1	2 h assay (*C. elegans*)	
Lantana rugosa Thunb	Leaf	Acetone	NT	At 1 mg/mL = 1	2 h assay (*C. elegans*)	McGaw and Eloff (2005)
Leonotis leonurus (L) R. BR	Leaf	Hexane	NT	At 1 mg/mL: – and –	2 h and 7-day assay (*C. elegans*)	McGaw et al. (2000)
		Ethanol	NT	At 1 mg/mL: –and ++		
		Water	NT	At 1 mg/mL: –and ++		
	Leaf	Water	NT	1.25	LDA (*H. contortus*)	Maphosa et al. (2010)
			NT	1.25	EHA (*H. contortus*)	

Table 3.1: Medicinal plants screened for anthelmintic activity against *Haemonchus contortus* and *Caenorhabditis elegans* used in the present study **Continued.**

Plant species	Part used	Solvent used	Activity		Type of assay and organism tested	Reference
			CA (mg/mL)	DBA (mg/mL)		
Leucosidea sericea Eckl. & Zeyh	Leaf	Acetone	NT	1.08	EHA (*H. contortus*)	Adamu et al. (2013)
			NT	1.27	LDA (*H. contortus*)	
	Leaf	Petroleum ether	0.52	NT	MLC (*C. elegans*)	Aremu et al. (2010)
		Dichloromethane	0.26	NT		
		Ethanol	0.26	NT		
		Water	8.33	NT		
	Stem	Petroleum ether	2.08	NT		
		Dichloromethane	4.17	NT		
		Ethanol	2.08	NT		
		Water	8.33	NT		
Searsia lancea (L.f.) F.A. Barkley	Bark	Hexane	NT	At 1 mg/mL = 2	2 h assay (*C. elegans*)	McGaw et al. (2007)
		Methanol	NT	At 2 mg/mL = 1		
	Leaf	Hexane	NT	At 0.5 mg/mL = 3		

CA- Colorimetric assay; DBA- Developmental and behavioural assay; MLC- Minimum lethal concentration; LDA-Larval development assay; EHA-Egg hatch assay; NT-Not tested; Activity symbols: (two symbols per activity represent results for two assays, that is 2 h and 7 days) - same number of nematodes as control; + small increase (about half number in control); ++ very small increase (less than 20% of control); In tested concentration meaning 1 = 80% of nematodes alive; 2 = 70% of nematodes alive; 3 = 60% of nematodes alive

3.7.1 *Caenorhabditis elegans*

The mode of action of anthelmintic drugs can be evaluated *in vitro* through nematode behaviour, locomotion and reproduction (Katiki et al., 2011). In the present study, the anthelmintic activity assessed via a nematode motility assay of extracts of the 10 plants is shown in **Table 3.2.** Effect of extracts on *C. elegans* was evaluated at three different concentrations (0.5, 1, and 2 mg/mL), similar to the method reported by McGaw et al. (2000) and McGaw and Eloff (2005). The activity of fractions was evaluated at 0.5 and 1 mg/mL and this was done to support the selection of the most active fraction at a low concentration for the isolation of compounds. The selected screening method employing an estimate of the percentage of motile nematodes after 24 and 48 h incubation was used as it facilitates comparison with previous work against PPN and to support the use of *C. elegans* as a model for bioassay-guided isolation in our study.

The percentage inhibition of juveniles motility increased with concentration, confirming the anticipated concentration-dependent response. Motility of *C. elegans* is as sensitive sublethal parameters such as reproduction and might be an indicator for neurobehavioral toxicity which offers possibility of a more rapid toxicity screening. To measure the degree of percentage immotility, we adopted the mortality approach which was proposed by Bogner et al. (2017), with categories which included inactive (0%); poor (0-25%); moderate (26-50%); good (51-71%) or strong (71-100%). Water and DCM/MeOH extracts showed good activity in reducing motility of juveniles and so did the acetone extract of *C. glabrum* (94%), *C. dentata* (88%) and *A. oppositifolia* (80%). Highest immotility of 100% was observed in all DCM/MeOH extracts at 1 mg/mL at all times of incubation. In some extracts of *C. anisata, C. orbiculata* and *C.*

glabrum, good activity was observed after 24 h, but further exposure resulted in some nematode juveniles recuperating after temporary paralysis. Water extracts of *H. pauciflorus*, *A. oppositifolia*, *L. sericea, S. lancea,* and *C. dentata* had strong effects on motility of juveniles, with inhibition ranging between 80 and 99% after 48 h. *Curtisia dentata* extracts had good activity at all incubation times, which could be due to the presence of betulinic acid, lupeol and ursolic acid, which were found to be active against *C. elegans* juveniles after 2 h and 7 days incubation (Shai et al., 2009). The strong activity shown by the water extract of *A*. *oppositifolia* (82%) disagrees with the report of Aremu et al. (2010) where a minimum lethal concentration of 4.17 mg/mL was required to kill *C. elegans* juveniles when using a colorimetric method. The moderate activity of *C. glabrum* (42%) water extract corroborates with findings of McGaw et al. (2000) when using behavioural methods **(Table 3.1)**. *Cotyledon orbiculata* water extract showed good activity which inhibited 72% of juveniles and this finding disagrees with the report of Aremu et al. (2010) when assayed using the colorimetric method **(Table 3.1).** Generally, all plant extracts prepared using the various solvents investigated showed good anthelmintic activity against *C. elegans*.

Although most extracts showed potential in inhibiting motility of *C. elegans* juveniles, fractionation was done on extracts of *C. anisata*, *L. leonurus*, and *L. rugosa*. These plants were selected based on their nematicidal activity (to be discussed in **Chapter 4**) when tested against *M. incognita*. Effects of fractions against *C. elegans* juveniles are shown in **Table 3.3**. All fractions at 1 mg/mL had good activity in inhibiting motility of *C. elegans*. At the lowest concentration of 0.5 mg/mL, good activity was observed with *C. anisata* (*n*-hexane, ethyl acetate and dichloromethane fractions), *L. rugosa* (ethyl acetate, dichloromethane and *n*-butanol fractions) and the *n*-hexane fraction of

L. leonurus at all incubation times. The good activity against nematode juveniles shown by these fractions at the lowest concentration was higher than that in the extracts, thus fractionation potentiated the activity of the fraction. This strengthens the rationale for isolating compounds responsible for activity in the plant from the active fractions.

Table 3.2: Percentage immotility (mean±SD) of selected plant extracts on *C. elegans* juveniles at different concentrations exposed for 24 and 48 h

Plant name	Concentration (mg/mL)	Percentage immotility (%					
		H_2O		Ace		DCM/MeOH	
		24 h	48 h	24 h	48 h	24 h	48 h
A. oppositifolia	0.5	11.24±4.85	**81.79±2.33**	42.14±3.71	**79.71±3.88**	**84.02±2.13**	**85.33±1.79**
	1.0	100.00±0.00	96.21±1.14	45.71±6.22	71.59±2.33	100.00±1.01	100.00±0.22
	2.0	100.00±0.00	100.00±0.00	68.57±4.12	60.00±3.66	100.00±0.00	100.00±0.00
C. anisata	0.5	41.59±4.11	24.00±3.92	19.47±4.35	22.40±2.44	**92.90±3.11**	56.17±4.50
	1.0	67.26±4.67	100.00±0.00	77.88±3.33	71.20±3.67	100.00±0.00	100.00±0.00
	2.0	100.00±0.00	100.00±0.00	90.27±0.17	100.00±0.00	100.00±0.00	100.00±0.00
C. glabrum	0.5	19.64±4.00	42.20±3.22	**78.07±3.11**	**93.82±2.10**	**100.00±0.00**	**84.55±2.11**
	1.0	100.00±0.00	62.43±3.87	92.11±2.17	99.23±1.09	100.00±0.00	100.00±0.00
	2.0	100.00±0.00	88.44±2.14	95.61±1.34	99.78±0.17	100.00±0.00	100.00±0.00
C. orbiculata	0.5	**79.65±2.44**	**72.29±3.14**	56.43±4.11	44.25±4.28	**71.95±3.11**	**83.52±2.55**
	1.0	95.56±2.10	93.95±2.47	86.25±2.88	79.79±3.22	94.90±1.74	97.51±0.33
	2.0	96.46±1.11	96.98±1.17	90.24±1.35	96.26±0.11	100.00±0.00	100.00±0.00
C. dentata	0.5	59.32±3.21	**81.36±2.57**	44.82±3.87	**87.53±1.78**	**84.17±2.01**	**90.18±2.10**
	1.0	97.93±2.37	94.46±2.11	99.85±0.91	99.87±0.78	97.93±1.04	100.00±0.00
	2.0	100.00±0.00	99.24±0.57	100.00±0.00	100.00±0.00	100.00±0.00	100.00±0.00
H. pauciflorus	0.5	**95.56±1.57**	**94.21±1.55**	30.77±4.67	N/A	**100.00±0.00**	**99.34±1.28**
	1.0	100.00±0.00	99.24±1.67	53.55±3.92	N/A	100.00±1.66	100.00±1.05
	2.0	100.00±0.00	100.00±0.00	33.43±2.22	51.13±3.25	100.00±0.00	100.00±0.00
L. rugosa	0.5	N/A	N/A	42.31±2.88	40.05±1.74	N/A	11.20±4.11
	1.0	46.90±3.88	12.80±5.10	56.21±3.87	73.55±3.20	100.00±0.00	100.00±0.00
	2.0	100.00±0.00	100.00±0.00	89.35±2.57	94.21±2.10	100.00±0.00	100.00±0.00
L. leonurus	0.5	**99.41±3.11**	67.51±3.25	60.36±3.17	22.17±5.11	**87.57±3.17**	**74.56±2.98**
	1.0	100.00±0.00	97.73±1.02	60.02±2.98	29.72±4.98	100.00±0.00	100.00±0.00
	2.0	100.00±0.00	100.00±0.00	90.24±1.98	96.73±1.08	100.00±0.00	100.00±0.00
L. sericea	0.5	**98.18±1.67**	**99.64±0.57**	17.14±3.47	32.75±4.89	**92.14±1.78**	**99.42±0.59**
	1.0	100.00±0.00	100.00±0.00	49.28±4.32	77.97±2.78	100.00±0.00	100.00±0.00
	2.0	100.00±0.00	100.00±0.00	80.71±2.24	98.26±1.00	100.00±0.00	100.00±0.00
S. lancea	0.5	**81.43±2.23**	**87.83±1.30**	39.64±4.67	65.36±3.88	**100.00±0.00**	**83.93±2.24**
	1.0	100.00±0.00	100.00±0.00	68.05±3.22	58.93±4.22	100.00±0.00	100.00±0.00
	2.0	100.00±0.00	100.00±0.00	90.24±1.57	45.71±4.33	100.00±0.00	100.00±0.00
Levamisole	10.0 µg/mL	43.20±1.07	70.53±0.17	43.20±1.07	70.53±0.17	43.20±1.07	70.53±0.17
	5.0 µg/mL	2.67±1.01	39.29±0.11	2.67±1.01	39.29±0.11	2.67±1.01	39.29±0.11

Ace- acetone, H_2O- water, DCM/MeOH-dichloromethane/methanol; in bold are the strongest inhibition of juveniles motility (values greater than 71%) at low concentration of 0.5 mg/mL.

Table 3.3: Anthelmintic activity (percentage immotility, mean±SD) of *L. leonurus*, *C. anisata*, and *L. rugosa* fractions on *C. elegans* juveniles exposed at 0.5 and 1 mg/mL

Fractions	Concentration (mg/mL)	*L. leonurus*		*C. anisata*		*L. rugosa*	
		Time (h)		Time (h)		Time (h)	
		24	48	24	48	24	48
Water	0.5	24.00±1.17	15.60±2.22	N/A	46.70±4.22	N/A	13.80±3.91
	1.0	100.00±0.00	38.20±5.22	71.20±3.55	66.70±2.21	64.80±3.18	67.10±2.13
Ethyl acetate	0.5	N/A	56.40±3.54	**76.80±1.92**	**82.70±2.17**	57.60±4.17	**79.60±3.10**
	1.0	23.20±3.27	81.30±2.11	81.60±3.21	90.20±1.98	95.20±2.11	93.70±2.88
n-Butanol	0.5	N/A	59.10±4.22	N/A	37.30±4.58	**91.20±2.58**	**92.40±0.92**
	1.0	97.60±2.21	96.00±2.00	81.60±3.41	77.30±3.27	79.20±2.22	90.70±1.00
Dichloromethane	0.5	N/A	44.00±3.14	**73.60±3.11**	**80.40±3.22**	48.80±2.22	**83.60±0.08**
	1.0	30.40±2.10	76.40±3.11	96.00±0.21	95.10±0.00	97.60±1.01	98.70±0.51
n-Hexane	0.5	**70.40±0.52**	**81.30±2.11**	**88.80±3.11**	**94.20±0.37**	66.40±1.99	69.30±1.25
	1.0	88.00±2.22	91.10±1.59	97.60±1.00	100.00±0.00	99.20±.00	98.20±1.00
Levamisole	5.0 ug/mL	4.00±2.31	43.56±1.99	4.00±2.31	43.56±1.99	4.00±2.31	43.56±1.99
	10.0 ug/mL	68.80±1.47	88.44±0.11	68.80±1.47	88.44±0.1	68.80±1.47	88.44±0.11

N/A: number of nematodes in the blank was higher than the one in the tested sample; in bold are the strongest inhibition of juvenile motility (values greater than 71%) at low concentration of 0.5 mg/mL

3.7.2 *Haemonchus contortus*

The ability of the extracts and/ or fractions to reduce larval hatchability is said to be epidemiologically beneficial as far as larval pasture contamination is concerned (Max, 2010). The lowest concentration tested in the present study was 0.10 mg/mL and all extracts showed good activity in inhibiting the L1 of *H. contortus* from hatching. All water extracts were able to inhibit larval hatchability above 60% of *H. contortus* at the highest concentration of 3.33 mg/mL (**Figure 3.1**). However, inhibition by *L. rugosa* at the same concentration was moderate with less than 40%. *Searsia lancea* water extract showed moderate activity (28%) at the lowest concentration (0.10 mg/mL) with weak activity shown by *L. rugosa* (13%). Acetone and DCM/MeOH extracts (**Figures 3.2** and **3.3** respectively) had good activity at all the tested concentrations. At the lowest concentration, *L. rugosa* acetone extract showed the highest larval inhibitory activity followed by *L. leonurus* and *C. anisata* with inhibition of 96, 77 and 60% respectively. The DCM/MeOH extract of *L. leonurus* inhibited 100% of larvae from hatching at the lowest concentration tested (**Figure 3.3**).

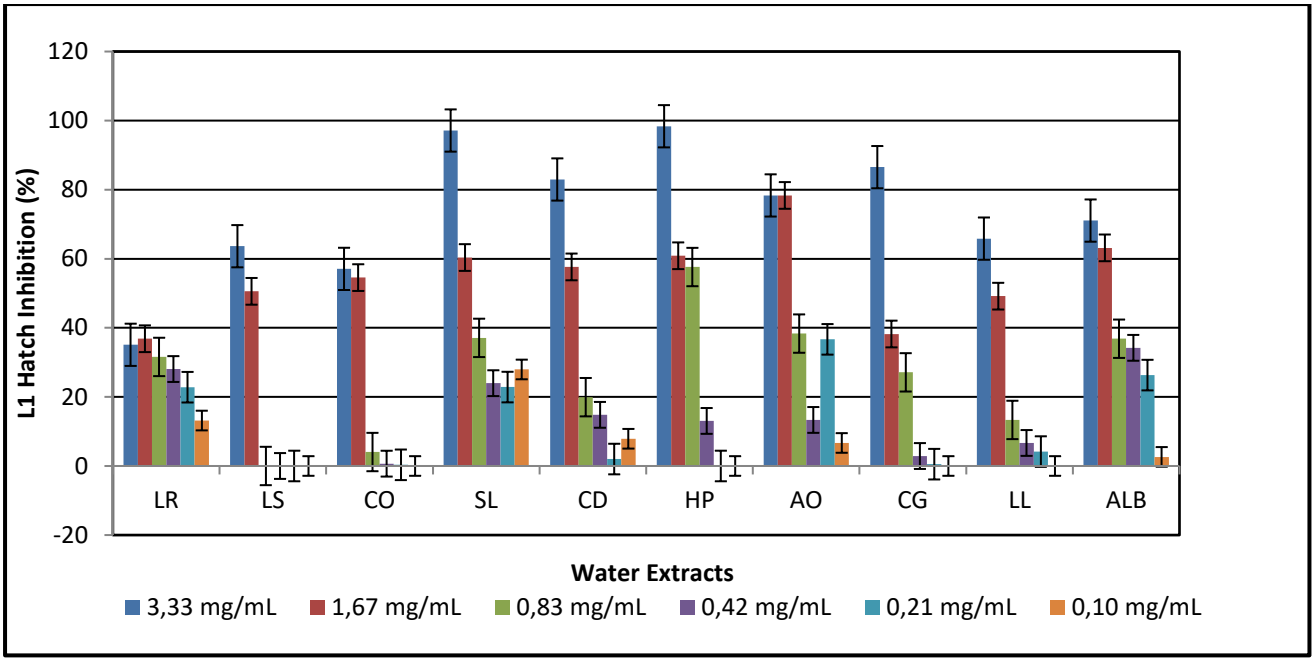


Figure 3.1: Inhibitory activity of water extracts on L1 hatching of *H. contortus*

Treatments: *Lantana rugosa* (LR), *Leucosidea sericea* (LS), *Cotyledon orbiculata* (CO), *Searsia lancea* (SL), *Curtisia dentata* (CD), *Hippobromus pauciflorus* (HP), *Acokanthera oppositifolia* (AO), *Clerodendrum glabrum* (CG), *Leonotis leonurus* (LL), and Albendazole (ALB)

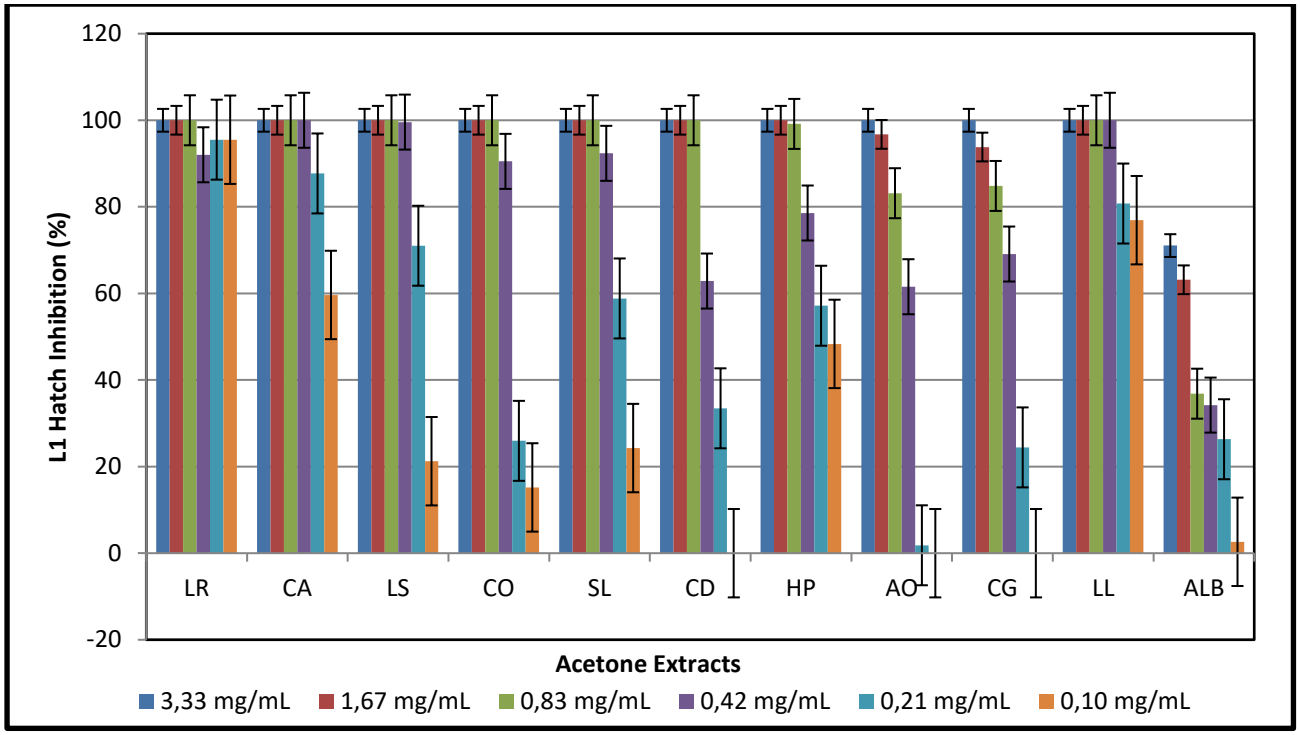


Figure 3.2: Inhibitory activity of acetone extracts on L1hatching of *H. contortus*

Treatments: *Lantana rugosa* (LR), *Leucosidea sericea* (LS), *Cotyledon orbiculata* (CO), *Searsia lancea* (SL), *Curtisia dentata* (CD), *Hippobromus pauciflorus* (HP), *Acokanthera oppositifolia* (AO), *Clerodendrum glabrum* (CG), *Leonotis leonurus* (LL), and Albendazole (ALB)

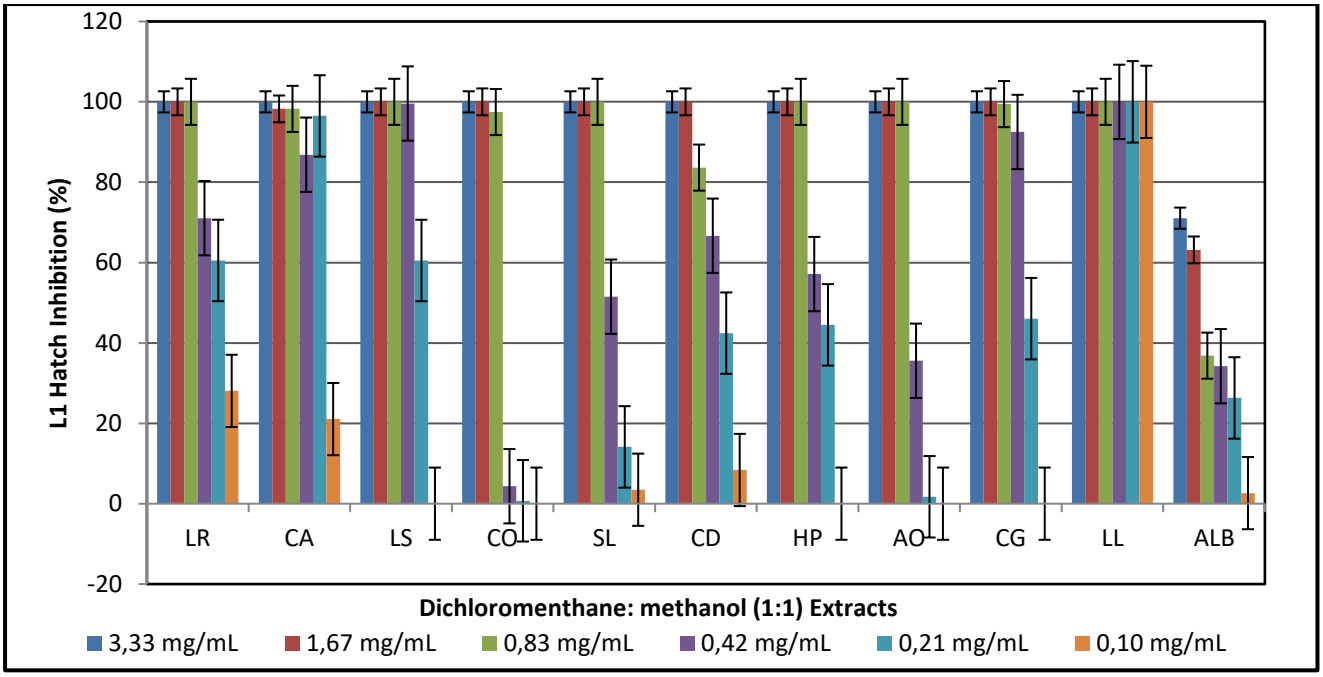


Figure 3.3: Inhibitory activity of dichloromethane: methanol extracts on L1 hatching of *H. contortus*

Treatments: *Lantana rugosa* (LR), *Leucosidea sericea* (LS), *Cotyledon orbiculata* (CO), *Searsia lancea* (SL), *Curtisia dentata* (CD), *Hippobromus pauciflorus* (HP), *Acokanthera oppositifolia* (AO), *Clerodendrum glabrum* (CG), *Leonotis leonurus* (LL), and Albendazole (ALB)

Table 3.4 represents the concentration of the extract required to inhibit 50% of *H. contortus* larvae from hatching. Potential candidates ranged from 0.03 to 0.1 mg/mL. *Leucosidea sericea* (acetone, DCM/MeOH extracts), *C. orbiculata* (acetone extract), *S. lancea* (acetone extract) and *H. pauciflorus* (acetone, DCM/MeOH extracts) had the lowest EC_{50} values. All the extracts of *L. rugosa* and *C. anisata* required concentrations lower than the tested range to effectively inhibit 50% of larvae from hatching. The good activity shown by the acetone extracts of *C. anisata*, *C. glabrum,* and *L. sericea* agrees with the findings by Adamu (2013) which reported LC_{50} values less than 2 mg/mL, although in the present study, values were less than 1 mg/mL. The

good activity of *L. sericea* could be due to the anthelmintic compounds previously isolated from the plant, namely aspidinol (Bosman et al. (2004), and agrimol G (Adamu, 2013). The *L. leonurus* water extract had an EC_{50} value of 0.84 mg/mL compared to 1.25 mg/mL obtained by Maphosa et al. (2010) and this difference could be due to the chemistry of the plants which can be affected by temperature and habitat. The good activity shown by *C. dentata* extracts in inhibiting the larvae from hatching supports the study done by Shai et al. (2009) where the acetone extract was active against larvae of *H. contortus*, that is, the plant is able to inhibit L1 hatching and also paralyse the larvae, making it a potential anthelmintic agent.

Table 3.4: Effective concentration (EC_{50} in mg/mL) of extracts from different plant species required to inhibit 50% of *H. contortus* larvae from hatching (mean±SE)

Plant name	Extracts		
	Water	Acetone	DCM/MeOH
L. rugosa	<0.10	<0.10	<0.10
C. anisata	<0.10	<0.10	<0.10
L. leonurus	0.81±0.26	0.44±0.13	N/A
L. sericea	2.35±0.01	**0.06±0.03**	0.14±0.02
C. orbiculata	3.16±0.14	0.16±0.01	0.52±0.02
C. glabrum	1.87±0.33	0.36±0.14	022±0.22
S. lancea	0.92±0.89	**0.04±0.03**	0.21±0.19
C. dentata	1.98±0.33	0.21±0.07	0.25±0.12
H. pauciflorus	0.79±0.00	**0.04±0.02**	**0.03±0.02**
A. oppositifolia	4.10±0.03	0.53±0.23	0.48±0.01
Albendazole		0.80±0.33	

N/A denotes that the EC_{50} could not be calculated because the extract inhibited 100% of L1 from hatching at all concentrations; < 0.1 denotes that the EC_{50} values were below the lowest concentration tested; values in bold represent relative low EC_{50}

Leonotis leonurus, *L. rugosa* and *C. anisata* fractions were further tested against larval hatchability of *H. contortus*. All fractions of *L. leonurus* performed the best, followed by

those of *C. anisata*. *L. leonurus* fractions showed good activity in inhibiting the larvae from hatching at all tested concentrations (**Figure 3.4)**. *n*-Butanol (10%) and dichloromethane (20%) fractions had the highest inhibition at 0.10 mg/mL, compared to other fractions of the same plant at the same concentration. At a high concentration of 3.33 mg/mL, all fractions of *L. leonurus* had inhibition above 70% with the exception of ethyl acetate which inhibited 48% of larvae from hatching. Dichloromethane, *n*-butanol and water fractions of *L. leonurus* had good inhibition at most concentrations compared to ethyl acetate and *n*-hexane fractions. The *C. anisata* dichloromethane fraction also showed good activity followed by *n*-butanol **(Figure 3.5**). The ethyl acetate fraction was only active at high concentrations (0.8 to 3.33 mg/mL) with inhibition of 26% at 0.8 mg/mL. Lower concentrations including those of water and *n*-hexane fractions (except at 3.33 mg/mL for *n*-hexane) had stimulatory effects on larval hatching. *Lantana rugosa* (**Figure 3.6**) *n*-butanol fraction displayed good activity at all concentrations, except for 0.10 mg/mL. The good activity was also observed with high concentrations of ethyl acetate (1.67 and 3.33 mg/mL) and water fractions at 3.33 mg/mL with more than 40% inhibition. Lower concentrations of this plant also stimulated larval hatching.

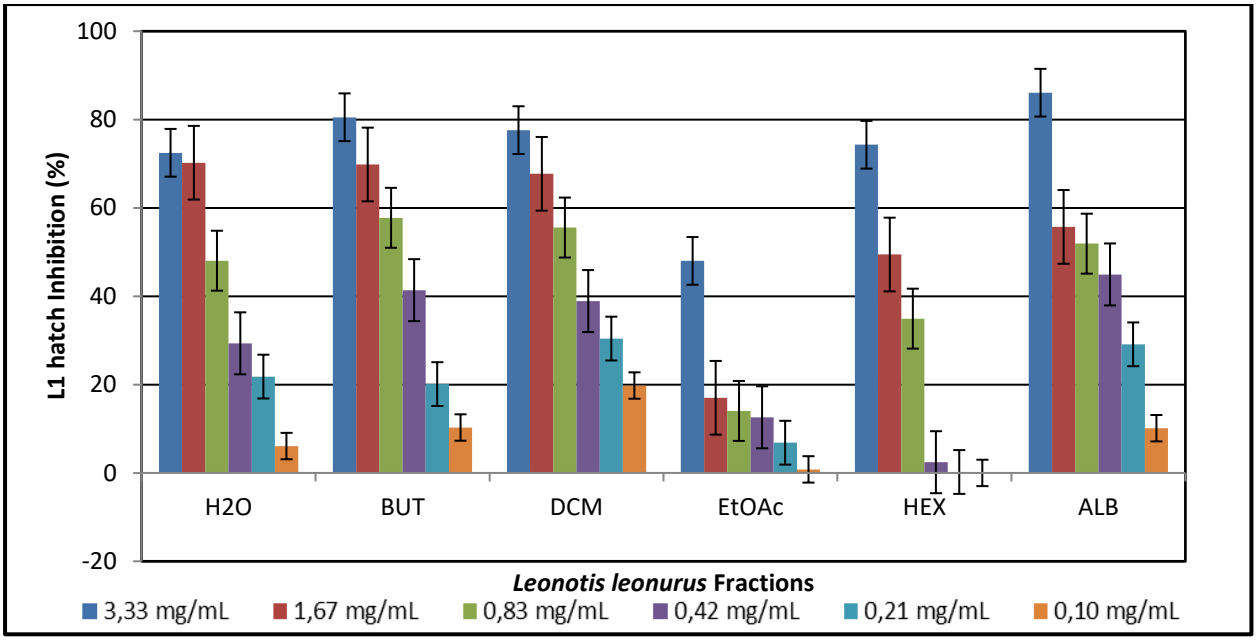


Figure 3.4: Inhibitory activity of *L. leonurus* fractions on L1 hatching of *H. contortus*

Inhibitory activity of *L. leonurus* fractions on L1 hatching of *H. contortus.* Treatments: Water (H_2O), *n*-Butanol (BUT), Dichloromethane (DCM), Ethyl acetate (EtOAc), *n*-Hexane (HEX), and Albendazole (ALB)

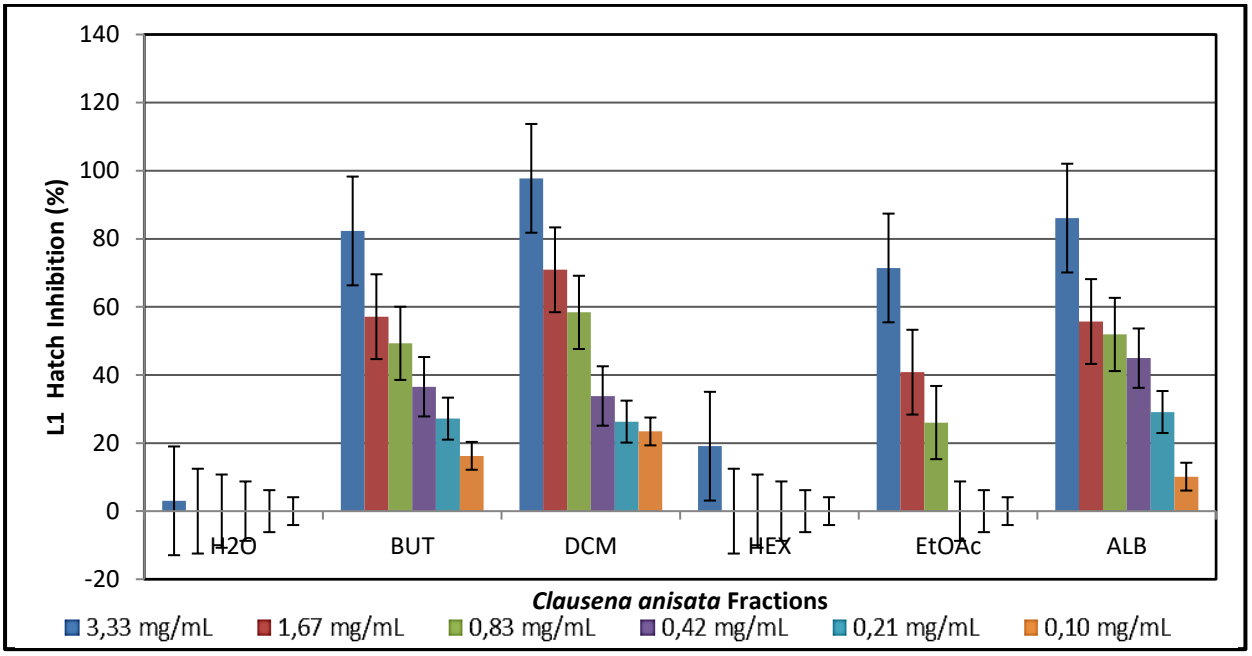


Figure 3.5: Inhibitory activity of *C. anisata* fractions on L1 hatching of *H. contortus*

Treatments: Water (H_2O), *n*-Butanol (BUT), Dichloromethane (DCM), Ethyl acetate (EtOAc), *n*-Hexane (HEX), and Albendazole (ALB)

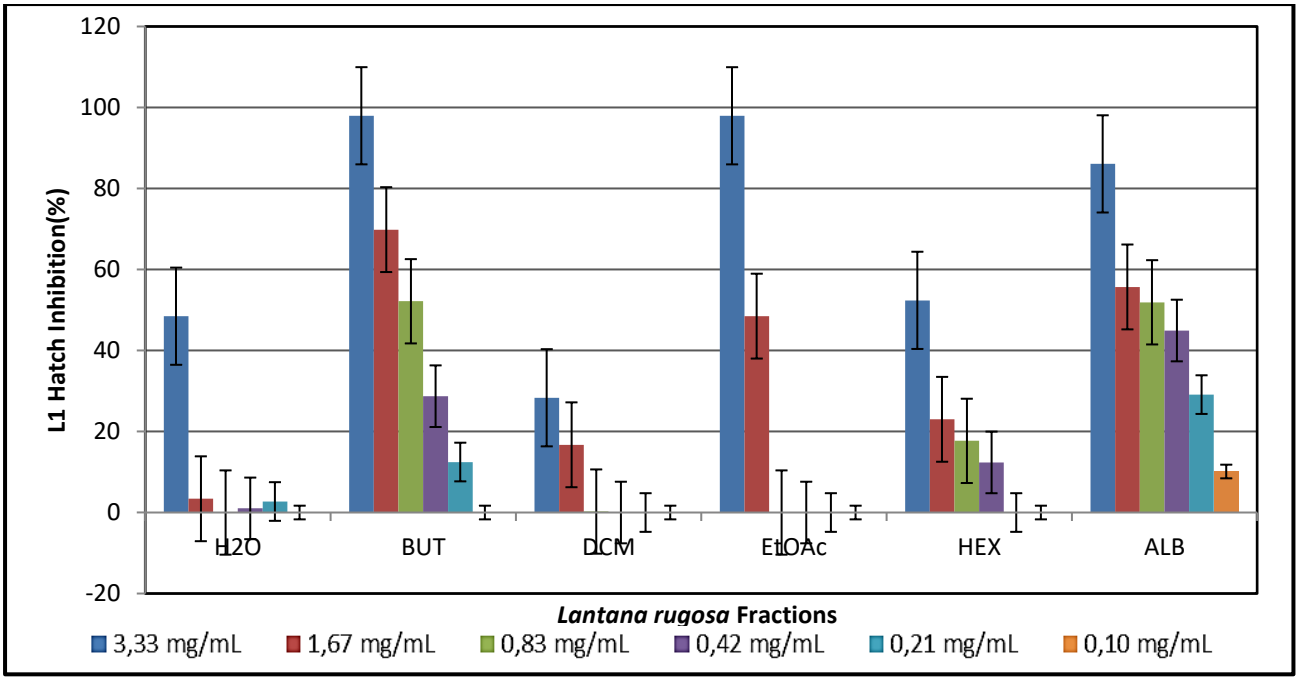


Figure 3.6: Inhibitory activity of *L. rugosa* fractions on L1 hatching of *H. contortus*

Treatments: Water (H_2O), *n*-Butanol (BUT), Dichloromethane (DCM), Ethyl acetate (EtOAc), *n*-Hexane (HEX), and Albendazole (ALB)

The good activity shown by the fractions is supported by the low EC_{50} values shown in **Table 3.5**. *Leonotis leonurus n*-butanol and dichloromethane fractions had the lowest EC_{50} values of 0.09 mg/mL and 0.19 mg/mL respectively. *n*-Hexane and water fractions of *C. anisata* were not active with EC_{50} values above 3.3 mg/mL (the highest concentration tested). Fractions were generally less effective than the extracts. The inactivity of the fractions in the *H. contortus* infective larvae hatch assay could therefore mean that fractionation could not potentiate the activity of fractions. The compounds in the plants most likely work in synergy since separation decreased activity.

Table 3.5: EC_{50} (mg/mL) of fractions from *L. leonurus*, *C. anisata* and *L. rugosa* in the L1 hatch assay

	EC_{50} (mg/mL)		
Fractions	***L. leonurus***	***C. anisata***	***L. rugosa***
Water	1.50±1.17	>3.33	3.88±0.05
n-Butanol	**0.09±0.27**	2.92±2.90	0.54±0.49
n-Hexane	0.72±1.50	>3.33	3.46±1.33
Dichloromethane	**0.19±0.10**	2.56±2.55	3.35±0.31
Ethyl acetate	2.61±3.40	2.24±0.08	1.83±0.24
Albendazole		0.64±0.27	

Albendazole = 0.64±0.27

>3.33 denotes that the EC_{50} values were higher the highest concentration tested

values in bold represent relative low EC_{50}

3.7.3 Cytotoxicity

It is important to test for the safety of plants which may pose a risk to humans or other animals. The cytotoxic effects of *L. leonurus*, *C. anisata* and *L. rugosa* were evaluated against African green monkey kidney cells. Renal cells were selected because kidneys are the main site of excretion in animals, there is a good blood supply to the kidneys, and they have a high metabolic capacity (Adamu et al., 2013; Fouche et al., 2016). A plant extract is considered to be highly cytotoxic when the LC_{50} is 20 µg/mL and below (Kuete et al., 2011). Results indicated that most of the extracts were relatively non-toxic to Vero cells compared to the positive control, doxorubicin (LC_{50} = 0.0125 mg/mL) except for *L. rugosa* extracts which were highly toxic to cells with LC_{50} values of less than 0.0075 mg/mL (lowest concentration tested). The water extract of *L. leonurus* and acetone extract of *C. anisata* were not toxic with LC_{50} values of 0.169 mg/mL and 0.178 mg/mL respectively which are higher than the predetermined cut-off point (20 µg/mL) reported by Kuete et al. (2011) **(Table 3.6)**.

Table 3.6: Cytotoxic effects of nine extracts from *C. anisata*, *L. rugosa* and *L. leonurus* on Vero monkey kidney cells (LC_{50} in mg/mL)

Plant name	Extracts	LC_{50} (mg/mL)
C. anisata	Water	0.063±0.005
	DCM:MeOH	0.065±0.012
	Acetone	**0.178±0.040**
L. rugosa	Water	<0.0075
	DCM:MeOH	<0.0075
	Acetone	<0.0075
L. leonurus	Water	**0.169±0.004**
	DCM:MeOH	0.052±0.003
	Acetone	0.072±0.006
Positive control	Doxorubicin	0.0125±2.320

DCM;MeOH = Dichloromethane: methanol; values in bold represent relative non-toxicity to cells

The safety of *L. leonurus* and *C. anisata* extracts on Vero cells has prompted further analysis where fractions from these two plants were also tested for cytotoxicity. **Table 3.7** shows the cytotoxicity results of the fractions against Vero cells. All tested fractions were not toxic to the cells when compared to doxorubicin, with water fractions having LC_{50} values above the highest concentration tested (0.1 mg/mL).

Table 3.7: Cytotoxic effects of *L. leonurus* and *C. anisata* and fractions on Vero monkey kidney cells (LC_{50} in mg/mL)

Fractions	LC_{50} (mg/mL)	
	L. leonurus	***C. anisate***
Water	**0.346±0.331**	**0.609±0.864**
n-Hexane	0.031±0.005	0.036±0.003
n-Butanol	0.030±0.004	0.025±0.001
Ethyl acetate	0.024±0.003	0.067±0.012
Dichloromethane	0.020±0.000	0.041±0.011
Doxorubicin	0.0102±0.0025	

values in bold represent relative non-toxicity to cells

3.7.3.1 Selectivity index

The selectivity index (SI) was calculated for *H. contortus* activity to identify the least toxic and most effective plants in terms of their anthelmintic activity against larvae of the parasite **(Table 3.8)**. The higher the value of the selectivity index, the safer the extract or fraction, with an SI value above 1 indicating greater biological activity than toxicity (Takaishi et al., 2008). Selectivity index is calculated by dividing the cytotoxicity LC_{50} by the EC_{50} obtained in the biological activity assay (Adamu et al., 2013). *Lantana rugosa* and *L. leonurus* extracts had SI index below 1. Only the extracts of *C. anisata* had SI values above 1. The lowest toxicity obtained with the extracts of *C. anisata* and SI index above 1 in the infective larvae hatch assay against *H. contortus* does not agree with the findings of Adamu et al. (2013) against the same cells and nematode species. The difference in activity could be due to the differences in concentration tested, or chemical variation in the source of plant material. The initial concentration used by Adamu and colleagues ranged from 12.50 mg/mL to 0.39 mg/mL while in the present study concentration it was from 3.33 mg/mL to 0.10 mg/mL. The resultant EC_{50} value of less than 0.01 mg/mL in the present study compared to 1.80 mg/mL in the study of Adamu et al. (2013) influenced the results significantly. For therapeutic drug development, lower concentrations are often advised to avoid toxicity and the use of a high quantity of material. The high selectivity index obtained with the extracts of *C. anisata* means that the plant has much better anthelmintic activity than toxicity to normal mammalian cells. The leaf extract of *C. anisata* was previously reported to be toxic to brine shrimp larvae (Makirita et al., 2016). When testing using the MTT assay, the acetone leaf extracts of *C. anisata* were reported to be safe by Adamu et al. (2013) and similar observations were made in the current study.

The low SI index for the extracts of *L. rugosa* is related to the high toxicity obtained against Vero cells, thus, although it had promising anthelmintic activity, the plant is unsuitable for further analysis since it is toxic to normal cells. The *L. leonurus* extract was not toxic to the Vero cells and had good anthelmintic activity, but the low SI index could mean that the plant might be potentially toxic when given to animals for treating helminth infection. Larval development inhibition was not tested in the present study, so it will be useful to compare the SI index in both assays. Several studies have been conducted in testing the safety of *L. leonurus* leaf extracts. Hurinanthan (2009) reported no toxicity to brine shrimp, which was also observed by McGaw et al. (2000). It was also non-toxic when tested on a myelogenous leukemia cell line and negative using the Ames *Salmonella* mutagenicity assay. El-Ansari et al. (2009) reported the plant to have hepatoprotective properties. The safety reports by these researchers support the *in vitro* safety of the plant in the current study when testing for cytotoxicity using the MTT assay. However, the low SI index when testing for larval hatch inhibition in the present study against *H. contortus* could potentially label the plant as unsafe for treating animals. The potential toxicity of the plant was also reported where an aqueous extract revealed a cytotoxic effect against HL-60 cells, an acute promyelocytic leukaemia cell line (Kee et al., 2008). Additionally, Maphosa et al. (2008) reported the toxicity of the plant on the rats in terms of hematological parameters and liver, lungs, heart, and spleen which can cause death.

Table 3.8: Selectivity index (SI) of the *C. anisata*, *L. leonurus* and *L. rugosa* extracts

Plant	Extract	SI (EHA)
C. anisata	Water	>1
	DCM/MeOH	>1
	Acetone	>1
L. leonurus	Water	0.21
	DCM/MeOH	N/A
	Acetone	0.16
L. rugosa	Water	<1
	DCM/MeOH	<1
	Acetone	<1

>1 denotes that SI is greater than 1 while <1 is lower than 1.

Selectivity index values of the fractions were also determined using larval hatchability assay results of *H. contortus*. Although the fractions were relatively non-toxic to the Vero cells, they all had SI values below 1 **(Table 3.9)** thus fractionation of the extracts did not enhance activity.

Table 3.9: Selectivity index of the fractions of *L. leonurus* and *C. anisata*

Fractions	SI Index	
	L. leonurus	***C. anisata***
Water	0.23	<1
n-Butanol	0.33	0.09
n-Hexane	0.04	<1
Dichloromethane	0.11	0.02
Ethyl acetate	0.01	0.03

<1 denotes that SI is lower than 1.

3.7.4 Correlation in anthelmintic activity

The anthelmintic activity of the plants against the tested nematodes was further analysed using correlation statistics. The plants investigated in the present study were evaluated for their nematicidal activity against PPN **(Chapter 4)**. Correlation statistics were applied to see if *C. elegans* can be used as a model organism for activity against plant and animal-parasitic nematodes. According to Pallant (2001), correlation analysis is the relationship between two continuous variables. It is used to describe the strength and direction of the linear relationship between continuous variables. Cohen (1988) suggested the interpretation of values as: weak where r = 0.0 to 0.29; moderate where r = 0.30 to 0.49 and strong correlation with r = 0.50 to 1.0. In the present study as shown in the correlation **Table 3.10**, there is a statistically significant (p = 0.004) perfect negative strong correlation (r = -0.516) between animal and free-living nematodes. Perfect negative correlation indicates that the two variables move in opposite directions with equal magnitude (Cohen, 1988). The strong correlation supports the use of *C. elegans* as a model for developing anthelmintic drugs from plants in the treatment of animal-parasitic nematodes (APN) (McGaw et al., 2000; McGaw and Eloff, 2005; McGaw et al., 2007; Shai et al., 2009; Aremu et al., 2010; Maphosa et al., 2010; Adamu et al., 2013). Plant-parasitic and free-living nematodes (FLN) demonstrated a perfect negative weak correlation at p = 0.031 (r = -0.276). According to Chitwood (2002), a poor correlation has been reported when using *C. elegans* as a model for developing nematicides against some phytoparasitic nematodes. In that report, high levels of broad-spectrum toxins were identified instead of phytoparasitic nematode-specific compounds. The correlation between APN and PPN was not statistically significant (p = 0.061), however moderate correlation was observed with r = 0.330. This could mean that plants with good activity against APN

can also be used in the management of PPN. From the present study, it was difficult to find good correlations between all tested organisms and this could be due to different concentrations used. It would probably be better to use similar concentrations and test for both J2 hatch and juvenile motility for PPN and L1 hatch and larval development for APN. In comparison with FLN, motility can be used employing different methods such as behavioural assays and colorimetric experiments.

Table 3.10: Correlation between animal, plant, and free-living nematodes for 10 plants extracted with three solvents exposed at different times at the lowest concentration tested

		Plants	**Exposure Time**	**Free-living nematodes at 0.5 mg/mL**	**Animal-parasitic nematodes at 0.104 mg/mL**	**Plant-parasitic nematodes at 1 mg/mL**
Plants	Pearson Correlation	1				
	Sig. (2-tailed)					
	N	99				
Exposure Time	Pearson Correlation	0.000	1			
	Sig. (2-tailed)	1.000				
	N	99	99			
Free-living nematodes at 0.5 mg/mL	Pearson Correlation	0.229	0.157	1		
	Sig. (2-tailed)	0.075	0.228			
	N	61	61	61		
Animal-parasitic nematodes at 0.104 mg/mL	Pearson Correlation	0.012	. a	-0.516**	1	
	Sig. (2-tailed)	0.947	0.000	0.004		
	N	33	33	30	33	
Plant-parasitic nematodes at 1 mg/mL	Pearson Correlation	-0.076	0.238*	-0.276*	0.330	1
	Sig. (2-tailed)	0.454	0.017	0.031	0.061	
	N	99	99	61	33	99

* Correlation is significant at the 0.05 level (2-tailed)

** Correlation is significant at the 0.01 level (2-tailed)

as Cannot be computed because at least one of the variables is constant (animal-parasitic nematodes results were read once after 48 hours)

3.7.5 Chemical profiling

Results showing chemical constituents in the acetone and DCM/MeOH extracts of ten South African plants are shown in **Table 3.11**. The profiled chemicals based on GC-MS analysis indicated the presence of various chemicals, including fatty acids. Only constituents with high percentage in the extract were reported with similarity above 85 percentage showing at certain retention time. Most chemicals were similar in most plants, to mention some: octadecenamide, 9-Octadecenamide, (Z)-, octadecanoic acid, dodecanamide, hexanamide, hexadecanoic acid and hexanoic acid. Similar compounds were detected with different extractants used per plant; however the quantity was not the same. For example, the *H. pauciflorus* DCM/MeOH extract contained a high quantity of hexanamide (11%) visible at retention time of 1078.2s while the acetone extract had 9% of the same compound showing at 1183.5s. Dodecanamide in the same plant was high in the acetone extract with 6% detectable at 1202.4s retention time while in the DCM/MeOH extract it was 4% at 1204.1s. This could mean that one should consider the solvent for extraction since certain chemicals dissolve better in solvents of similar polarity. Similar trends were observed in other plants, for example 9-octadecenamide, (Z)- was high in the DCM/MeOH extract of *A. oppositifolia* with 28% appearing at retention time of 1183.4s and low in the acetone extract with 16% at 1184,2s. Most chemicals even so the amount was not the same, they were mostly detected in same time while in some the distance was not far. n-Hexadecanoic acid, one of the most ubiquitous chemicals detected in almost all plants is commonly known as palmitic acid. It has been reported to have nematicidal, pesticidal and antioxidant properties (Komansilan et al., 2012). There are other chemicals which have been reported to have pharmacological activity, such as 9,12-octadecadienoyl chloride, (Z, Z)-. According to Vijayabaskar and Elango (2018), 9,12-

octadecadienoyl chloride, (Z,Z)- (linoleoyl chloride) is known to have nematicidal, anticancer, hepatoprotective as well as insectifugal properties. Antibacterial and anti-inflammatory activity has been reported with 9-octadecenamide, (Z)- (Hussein et al., 2016). The presence of these chemicals, even those occurring in lesser amounts, may contribute to the anthelmintic activity of the plants in synergism with other major compounds detected. Compounds detected in the plant with previous reports of nematicidal activity also support the anthelmintic activities reported in the present study.

Table 3.11: Chemical profiling of acetone and DCM/MeOH extracts from ten South African plants

Plant name	Extract	Chemical name	Weight	Formula	CAS	Similarity %	Retention time (s)	Area %
A. oppositifolia	Acetone	Dodecanamide	199	$C_{12}H_{25}NO$	1120-16-7	88	1075.6	9
		9-Octadecenamide, (Z)-	281	$C_{18}H_{35}NO$	301-02-0	92	1184.2	16
	DCM/MeOH	Dodecanamide	199	$C_{12}H_{25}NO$	1120-16-7	91	1075.5	9
		9-Octadecenamide, (Z)-	281	$C_{18}H_{35}NO$	301-02-0	92	1183.4	28
C. anisata	Acetone	n-Hexadecanoic acid	256	$C_{16}H_{32}O_2$	57-10-3	92	961.3	5
		Octadecanoic acid (stearic acid)	284	$C_{18}H_{36}O_2$	57-11-4	93	1095.1	7
		9,12,15-Octadecatrienal	262	$C_{18}H_{30}O$	26537-71-3	86	1346	3
		Dotriacontane	450	$C_{32}H_{66}$	544-85-4	94	1598.1	3
		Eicosane	282	$C_{20}H_{42}$	112-95-8	90	1346.8	3
	DCM/MeOH	Benzothiazole	135	C_7H_5NS	95-16-9	95	328.3	5
		Octadecanoic acid	284	$C_{18}H_{36}O_2$	123-94-4	91	1093.6	5
C. glabrum	DCM/MeOH	Hexanamide	115	$C_6H_{13}NO$	628-02-4	87	1076.7	11
		9-Octadecenamide, (Z)-	281	$C_{18}H_{35}NO$	301-02-0	92	1183.7	22
		Dodecanamide	199	$C_{12}H_{25}NO$	1120-16-7	92	1202.9	7
C. orbiculata	Acetone	Neophytadiene	278	$C_{20}H_{38}$	504-96-1	88	846.2	3
		9-Octadecenamide, (Z)-	281	$C_{18}H_{35}NO$	301-02-0	93	1186.4	25
		Dodecanamide	199	$C_{12}H_{25}NO$	1120-16-7	93	1204.5	8
	DCM/MeOH	9-Octadecenamide, (Z)-	281	$C_{18}H_{35}NO$	301-02-0	92	1183.3	22
		Dodecanamide	199	$C_{12}H_{25}NO$	1120-16-7	89	1202.2	7
		Dotriacontane	450	$C_{32}H_{66}$	544-85-4	90	1667.9	9
C. dentata	Acetone	9-Octadecenamide, (Z)-	281	$C_{18}H_{35}NO$	301-02-0	92	1183.5	14
		Dodecanamide	199	$C_{12}H_{25}NO$	1120-16-7	94	1202.5	4
	DCM/MeOH	Dodecanamide	199	$C_{12}H_{25}NO$	1120-16-7	92	1076.3	13
		9-Octadecenamide, (Z)-	281	$C_{18}H_{35}NO$	301-02-0	92	1184.2	33

Table 3.11: Chemical profiling of acetone and DCM/MeOH extracts from ten South African plants **Continued**.

Plant name	Extract	Chemical name	Weight	Formula	CAS	Similarity %	Retention time (s)	Area %
H. pauciflorus	Acetone	Hexanamide	115	$C_6H_{13}NO$	628-02-4	86	1183.5	9
		Dodecanamide	199	$C_{12}H_{25}NO$	1120-16-7	92	1202.4	6
		Thunbergol	290	$C_{20}H_{34}O$	25269-17-4	85	1661.5	18
		9-Octadecenamide, (Z)-	281	$C_{18}H_{35}NO$	301-02-0	92	1183.5	26
	DCM/MeOH	Hexanamide	115	$C_6H_{13}NO$	628-02-4	88	1078.2	11
		9-Octadecenamide, (Z)-	281	$C_{18}H_{35}NO$	301-02-0	93	1185.8	25
		Dodecanamide	199	$C_{12}H_{25}NO$	1120-16-7	95	1204.1	4
L. rugosa	Acetone	Octadecanoic acid	284	$C_{18}H_{36}O_2$	64165-21-5	93	1093.6	5
		Benzothiazole	135	C_7H_5NS	95-16-9	96	327.1	3
		2,5-Dimethyl-2-(2-tetrahydrofuryl)tetrahydrofuran	170	$C_{10}H_{18}O_2$	0-00-0	85	615.2	4
		à-Amyrin	426	$C_{30}H_{50}O$	638-95-9	86	1680.3	3
L. leonurus	DCM/MeOH	Dodecanamide	199	$C_{12}H_{25}NO$	1120-16-7	90	1080.5	6
		9-Octadecenamide, (Z)-	281	$C_{18}H_{35}NO$	301-02-0	92	1188.1	20
L. sericea	Acetone	9-Octadecenamide, (Z)-	281	$C_{18}H_{35}NO$	301-02-0	92	1196.8	41
		Dodecanamide	199	$C_{12}H_{25}NO$	1120-16-7	90	1215.3	14
	DCM/MeOH	Hexanamide	115	$C_6H_{13}NO$	628-02-4	88	1076.1	18
		9-Octadecenamide, (Z)-	281	$C_{18}H_{35}NO$	301-02-0	92	1184	45
		Dodecanamide	199	$C_{12}H_{25}NO$	1120-16-7	90	1202.7	13
S. lancea	Acetone	9-Octadecenamide, (Z)-	281	$C_{18}H_{35}NO$	301-02-0	92	1184.7	26
		Dodecanamide	199	$C_{12}H_{25}NO$	1120-16-7	95	1202.4	7
		1-Iodo-2-methylundecane	296	$C_{12}H_{25}I$	73105-67-6	92	1482.9	4
		Dotriacontane	450	$C_{32}H_{66}$	544-85-4	90	1578.5	4
	DCM/MeOH	9-Octadecenamide, (Z)-	281	$C_{18}H_{35}NO$	301-02-0	91	1183.3	37
		Dodecanamide	199	$C_{12}H_{25}NO$	1120-16-7	91	1202.2	10
		Dotriacontane	450	$C_{32}H_{66}$	544-85-4	91	1482.7	5

Conclusion

In conclusion, all extracts prepared with solvents of varying polarities had anthelmintic activity against *C. elegans* and *H. contortus*. The anthelmintic activity of *A. oppositifolia*, *S. lancea*, *C. orbiculata*, *H. pauciflorus*, and *L. rugosa* is reported for the first time against *H. contortus*. The presence of various chemicals present in all the plants might havecontributed towards the activity against *C. elegans* and *H. contortus*. The *in vitro* safety of the plant extracts was only tested against one cell line in selected concentrations, and therefore further toxicity tests on other cell lines as well as *in vivo* assays for promising candidates are recommended to confirm the safety of the plants. There was a good relationship between activity against APN and FLN compared to PPN. A moderate correlation between activity against FLN and PPN means that *C. elegans* may be useful as a model in finding nematicidal agents from plants.

Gas chromatography-mass spectroscopy analysis provided a useful guide to the chemical composition of the extracts, identifying a number of compounds in the extracts with known bioactivities. Perhaps other techniques such as liquid chromatography–mass spectrometry (LC-MS), capillary electrophoresis–mass spectrometry (CE-MS), and nuclear magnetic resonance (NMR) spectroscopy may be employed in the future to detect other diverse metabolites including less volatile compounds.

CHAPTER 4

Investigating the potential of South African plants as crop protective agents against soilborne pathogens infecting tomatoes

4.1 Introduction

Diseases caused by soilborne pathogens affect both production and development of crops. Seedling, root rot and vascular diseases are among the important diseases caused by soilborne pathogens (Katan, 2017). Soilborne pathogens include nematodes, fungi, bacteria and viruses which are microscopic, difficult to control and costly (Matthiessen and Kirkegaard, 2006). Parasitic nematodes of plants and animals are economically important, causing great losses in agricultural production (Abdel-Rahman et al., 2013). Vegetables provide important sources of nutrition in the human diet, and their production improves the livelihoods of smallholder and commercial farmers (Ngegba et al., 2016). Most vegetables are susceptible to plant-parasitic nematodes (PPN), in particularly tomato, melon, carrot, aubergine, lettuce, cucumber and pepper (Abiola, 2020). According to Abad and colleagues, annual agricultural losses due to PPNs has been estimated to be $157 billion (Abad et al., 2008).

Meloidogyne spp. are among the most important root-knot nematodes (RKN) genera affecting the quantity and quality of production of many annual and perennial crops (Pavaraj et al., 2012). Symptoms of nematode infection in plants include signs of chlorosis, stunted growth, root lesions as well as root galling. These signs have been reported to be similar to those of nutrient deficiencies, in particular of nitrogen

deficiency (Osei et al., 2011; Nguyen et al., 2018). According to Collange et al. (2011), one of the most important signs of infection is root galling, which can be easily seen by farmers.

Meloidogyne infection affects translocation of water and nutrients in the root system and some phytopathogens such as bacteria and fungi may enter the xylem and disrupt the movement of water, thus causing extensive damage to the crop (Jahr et al., 1999). Bacterial diseases caused by *Clavibacter, Ralstonia* and *Xanthomonas* species are a major concern in the production of tomato and other crops. *Clavibacter michiganensis* subsp. *michiganensis* was reported by Wallis (1977) cited in Jahr et al. (1999) as the causal agent of bacterial canker and wilt in tomato which occurs through wounds, followed by invasion of the xylem vessels, which establishes a systemic vascular disease. The *Ralstonia solanacearum* species complex is comprised of three species: *R. solanacearum*, *R. pseudosolanacearum* and *R. syzygii* (Prior et al., 2016). *Ralstonia solanacearum* has a wide host range and infects crops in the vascular stems, thus resulting in a brown discoloration and drops of white or yellowish bacterial ooze that may be visible if the stem is cut (Singh and Siddiqui, 2012). *Ralstonia solanacearum* and *R. pseudosolanacearum* cause bacterial wilt and are major destructive pathogens in tomato production (Prior et al., 2016). Bacterial spots of tomatoes are caused by different *Xanthomonas* spp. such as *X. vesicatoria* and *X. perforans*, and these species have been reported to reduce the yield of tomatoes. The weight of harvested fruit in one study was reduced by 52% due to the defoliation of tomato leaves by *X. vesicatoria* (Reddy et al., 2012). Also, pathogenic fungi are major infectious agents in crops, causing alterations during developmental stages including post-harvest (Dellavalle et al., 2011).

Fusarium wilt caused by *Fusarium oxysporum* f. sp. *lycopersici* is an important disease of tomato (*Solanum lycopersicum*). Fusarium wilt has a synergistic relationship with *M. incognita* (Agbenin and Marley, 2006) and can cause high yield loss due to the disease complex and may also lead to breaking of resistance (Khpalwak, 2012). It causes deterioration in the root, the basal stem and thus results in the wilting of vegetable plants (Ignjatov et al., 2012). Crop losses due to diseases affect both food production and food security globally (Pavaraj et al., 2012). Phytopathogens, together with RKN, reduce both the quality and quantity of tomatoes, thus hindering agricultural production and the delivery of sustainable food.

Pesticides have been universally considered as the most efficient solution to control crop diseases. Nematicides are effective in reducing RKN infection, but they are not always cost efficient and are economically viable only for high value crops (Mandal and Nandi, 2013). Several active ingredients including methyl bromide, dibromochloropropane, ethylene dibromide and aldicarb (Temik) have been banned in various parts of the world due to their environmental and human toxicity (Abdel-Rahman et al., 2008). Moreover, their continued use can lead to some level of resistance in PPN species (Onkendi et al., 2014). According to Obradovic et al. (2008), streptomycin and copper-based compounds are effective antibiotics which have been used for controlling bacterial spots of tomatoes. Copper-containing bactericides have proven to be an effective preventive treatment against many bacterial diseases, mostly leaf spots and blights. Fungicides have been used successfully to protect plants against fungal attack. Excessive and unsystematic applications of these synthetic chemicals pose a threat to the environment and humans and some phytopathogens have developed resistance to such chemicals, making them ineffective (da Silva et al.,

2016). In addition, the associated costs and contamination of crops with chemical residues has necessitated the search for alternative methods to control the plant diseases.

Plant-derived chemicals offer a valuable alternative to such problems since they may be safer for humans, animals and the environment. According to Chitwood (2002), the US Environmental Protection Agency needs less data to register phytochemicals than to register conventional pesticides, and registration costs are lower, hence medicinal plants are a potentially viable alternative. Plant-derived metabolites may be considered as alternatives to pesticides, either as plant extracts, formulated phytochemicals or as organic amendments to soil (Chitwood, 2002).

Plant and animal-parasitic nematodes share many common and highly conserved biological processes with the free-living soil rhabditid nematode *Caenorhabditis elegans* (Abdel-Rahman et al., 2013). Several studies have been conducted to screen plants for use as anthelmintic agents using *C. elegans* as a model. According to Chitwood (2002), natural products active against mammalian parasites can serve as useful sources of compounds for examination of activity against plant parasites. *Caenorhabditis elegans* has also been used to provide insight into mechanisms for controlling PPN (Costa et al., 2009; Holden-Dye and Walker, 2014). The following chapter is aimed at investigating plants reported in **Chapter 3** for their ability to control RKN and phytopathogenic bacteria and fungi infecting tomatoes.

4.2 Materials and Methods

4.2.1 Plant collection and extract preparation

The leaves of the plant species were collected and extracted as described in **Section 3.2.1.1** and **3.2.1.2**. The plants collected were *Leonotis leonurus, Clausena anisata*, *Hippobromus pauciflorus*, *Leucosidea sericea*, *Lantana rugosa*, *Clerodendrum glabrum*, *Curtisia dentata*, *Acokanthera oppositifolia*, *Searsia lancea* and *Cotyledon orbiculata*. The extracts were then resuspended with 10% DMSO for the acetone and MeOH/DCM extracts, and sterile distilled water for water extracts and prepared according to the concentrations required for the subsequent experiments.

4.2.2 *Meloidogyne incognita* trials

4.2.2.1 Preparation of Meloidogyne incognita inoculum

A population of *M. incognita* race 2, confirmed by SCAR-PCR (Zijlstra, 2000; Fourie et al., 2001) was obtained from the Agricultural Research Council (ARC)-Grain Crops Institute, Potchefstroom, SA and propagated at ARC-TSC (Tropical and Subtropical Crops), Mbombela, SA. Nematode eggs and infective second-stage juveniles (J2) were extracted from the tomato roots. The root systems were gently removed from the soil, cleaned under running tap water, chopped into 1 cm pieces and agitated for 4 min in 300 mL of 1% NaOCl solution to release the eggs and J2 juveniles from the plant tissues (Hussey and Barker, 1973). The suspension was poured through a set of nested sieves with apertures from top to bottom of 150, 63, 38 and 25 µm. Eggs contained in the 38 and 25 µm aperture sieve were combined and washed with distilled water and collected for the egg inhibition experiment. For the second-stage juvenile (J2) motility experiment, unhatched J2 (contained in the 25 µm sieve) were incubated

at 25˚C for 7 days. After incubation, hatched J2 were washed from the container with distilled water and collected in the 25 µm aperture sieve.

4.2.2.2 Meloidogyne incognita J2 motility and hatch assay

The *in vitro* assays for the different concentrations were carried out in the laboratory of the Phytomedicine Programme, Department of Paraclinical Sciences, University of Pretoria. The J2 motility assay was carried out to determine the anthelmintic effect of the selected plant extracts to select the most active plant species. Two experiments were conducted for juvenile motility: the first experiment aimed to screen all plant extracts at a single concentration of 1 mg/mL, while the second experiment aimed to screen active plants at 10 different concentrations (0.1 mg/mL, 0.2 mg/mL, 0.3 mg/mL, 0.4 mg/mL, 0.5 mg/mL, 0.6 mg/mL, 0.7 mg/mL, 0.8 mg/mL, 0.9 mg/mL and 1 mg/mL) for both juvenile motility as well as J2 hatch inhibition (Khosa, 2013). Modification of the J2 hatch assay was done following the method described by Nguyen et al. (2018). Approximately 38 000 J2 in 10 mL water were used to carry out the motility assay and the same number of eggs was also used for the J2 hatch experiment with continuous agitation every 5 s. A total volume of 100 µL was obtained in both experiments by adding 10 µL of extracts and 90 µL containing 100 ± 20 J2 for juvenile motility and 100 ± 20 eggs for the J2 hatch assay. Plates were agitated for proper mixing and incubated at 22˚C in the dark. After 24, 48 and 72 h, the total number of motile and immotile J2 in each well were counted using an inverted compound microscope at 10× 40 magnification. For the J2 hatch assay, plates were incubated in an incubator at 25 ± 2°C and the number of hatched J2 was counted after 7, 10 and 14 days using an inverted compound microscope at 10× 40 magnification. The first motility assay was conducted with six replicates and repeated twice, while for different concentrations as

well as the J2 hatch assay, the experiment was done in triplicate and repeated twice. Distilled water was used as the negative control for water extracts while 10% DMSO (used to solubilise the organic extracts) was used as the control for the organic solvents. As positive control, 2 mg/mL of salicylic acid was used.

4.2.3 Antimicrobial experiments

4.2.3.1 Test microorganisms and growth conditions

Five pathogenic bacterial species: *Clavibacter michiganensis* subsp. *michiganensis* BD 1377 (*Cmm*), *Xanthomonas vesicatoria* BD 1349, *Xanthomonas perforans* BD 1346*, Ralstonia solanacearum* CBD 261*, Ralstonia pseudosolanacearum* BD 1443 and one fungal strain of *Fusarium oxysporum* f.sp. *lycopersici* PPRI 5457 were used in this study. These strains had previously been isolated from diseased tomato plants and were stored in the Plant Pathogenic and Plant Protecting Bacteria Culture Collection (verified by Goszczynska, T., 2018) and the National Collection of Fungi (verified by Venter, J. A., 2018) of the ARC-Plant Protection Research (ARC-PPR), Roodeplaat, South Africa.

Purified colonies of *Ralstonia*, *Xanthomonas* and *Cmm* were inoculated into Mueller Hinton broth (Beef infusions solids 2g/l; Starch 1.5g/l: Casein hydrolysate 17.5 g/l at pH 7.4 ±0.2) and incubated at 28±2°C for 24 h in an orbital shaker incubator with the latter incubated for seven days. *X. perforans* was incubated at 28±2°C for 10 days. *Fusarium* was inoculated into Potato Dextrose broth and incubated at 28±2°C for 48 h in an orbital shaker incubator.

4.2.3.2 Minimal inhibitory concentration (MIC) evaluation against bacterial and fungal strains

A serial microplate dilution method described by Eloff (1998a) was used to determine the minimal inhibitory concentrations of the crude extracts on the selected bacterial and fungal pathogens. The acetone and aqueous extracts were re-dissolved in their respective solvents while dichloromethane: methanol extracts were re-dissolved in 10% DMSO to give a concentration of 10 mg/mL. For the antibacterial assay, inoculum suspension from overnight cultures was prepared by diluting the bacterial colonies with fresh Müller Hinton broth to equate to McFarland standard No 1 (equivalent to $3.0x10^8$cfu/mL). For the antifungal assay inoculum, suspension from overnight cultures was prepared by diluting the fungal colonies with fresh Potato Dextrose broth to match a McFarland standard No 1 (equivalent to $3.0x10^8$cfu/mL). Sterile water (100 µL) was dispensed in each well of a 96-well microtitre plate and plant extracts (100 µL) were serially diluted two-fold down the columns. A 100 µL aliquot of bacterial or fungal culture was added to each well resulting in concentrations ranging from 2 500 µg/mL to 19.5 µg/mL. A similar two-fold serial dilution of streptomycin (Sigma) (starting concentration 2 000 µg/mL) was used as a positive control for the bacterial assay and amphotericin B (Sigma) (10 000 µg/mL) was the positive control for the fungal assay. The solvent dilutions were used as negative controls. For the antibacterial assay, plates were incubated for 24 h at 28°C in a humidified atmosphere. In the antifungal assay, plates were incubated for 48 h. To measure growth, *p*-iodonitrotetrazolium violet (INT) (40 µL of a 0.2 mg/mL solution in sterile water) was added to each well and incubated further for 30 min (against bacterial strains), 24 and 48 h (against fungal strains). The minimal inhibitory concentration was recorded where clear zones

indicated the inhibition of the tested bacteria by the extract. The reduction of INT to red formazan indicated bacterial growth.

4.3 Data collection and data analysis

The experiment for larval motility and J2 hatch against *M. incognita* was done in triplicate and repeated twice. Calculations were done as follows:

Percentage immotility was calculated using Abbott's formula (Abbott, 1925) as follows:

Immotility (%) = [**(**immotility percentage in treatment - immotility percentage in untreated control)/ (100-immotility percentage in untreated control)] ×100%.

The percentage of unhatched J2 in the hatch inhibition assay was calculated according to the following formula (Nguyen et al., 2013):

Hatch inhibition (%) = [(percentage unhatched J2 in untreated control- percentage unhatched J2 in treatment)/percentage unhatched J2 in untreated control] ×100.

Minimum Inhibitory Concentration (MIC) experiments were done in triplicate and repeated two times. Results are presented as the mean MIC value ±SD.

4.4 Results and Discussion

4.4.1 *Meloidogyne incognita*

The results for motility inhibition on *M. incognita* J2 of the extracts at 1 mg/mL are shown in **Table 4.1**. As reported by Galbieri and Belot (2016) cited in Ebone et al. (2019), there are several factors that affect the performance of nematicides, including the stage of development of the nematode, environmental temperature and the nature of the chemicals among others. Water and acetone extracts were more effective in inhibiting motility of the J2s compared to the DCM/MeOH extracts. Strong activity was observed with the acetone extract of *C. anisata* and the water extract of *L. leonurus,*

as immotility was more than 80% after 24 and 48 h of incubation. However, motility was reduced after 72 h incubation with both extracts, implying that only temporary paralysis was induced, and the juveniles were able to repercurate. Good activity was observed after 72 h of exposure to the DCM/MeOH extract of *L. leonurus* with immotility of 64% and strong inhibition of motile juveniles of 74% with the DCM/MeOH extract of *C. anisata*. Degree of motility was measured based on the mortality category described by Bogner et al. (2017). Moderate activity was observed with the extracts of *C. dentata*, *C. glabrum* and *C. orbiculata.* Immotility of more than 60% was also observed with the extracts of *L. rugosa* after 72 h.

Table 4.1: Effect of plant extracts on motility of *Meloidogyne incognita* juveniles (J2s) exposed at different times to 1 mg/mL of plant extracts (mean±SEM)

	24 Hours			48 Hours			72 Hours		
Treatments	**Water**	**Acetone**	**DCM/MeOH**	**Water**	**Acetone**	**DCM/MeOH**	**Water**	**Acetone**	**DCM/MeOH**
Blank		6.20±1.10			9.50±1.29			9.20±1.92	
Salicylic acid		61.75±5.06			75.00±7.55			78.50±6.36	
A. oppositifolia	6.00±1.58	7.00±1.22	8.50±2.08	6.75±0.96	4.83±1.47	7.00±0.82	14.40±5.32	17.20±2.28	13.50±2.38
C. anisata	29.50±6.69	**82.75±2.06**	20.40±4.72	**67.00±8.25**	**92.40±2.51**	20.20±3.56	**64.00±4.73**	**88.67±4.04**	**74.00±5.05**
C. glabrum	11.00±1.00	12.80±3.03	10.20±1.79	14.60±1.34	17.40±3.85	7.33±1.63	27.50±2.38	33.40±3.05	28.40±4.04
C. obiculata	13.00±1.41	10.33±1.75	13.20±3.03	20.00±3.90	19.00±3.54	24.75±0.96	26.25±2.63	18.75±2.99	26.33±2.31
C. dentata	9.50±2.07	12.17±2.40	9.17±1.47	14.50±2.65	10.75±0.69	18.25±2.36	30.00±3.16	32.40±3.85	32.40±4.34
H. pauciflorus	6.00±1.67	6.20±1.48	5.83±1.47	4.75±0.96	3.50±1.05	5.00±2.16	15.50±1.00	19.40±3.65	17.00±2.35
L. rugosa	19.33±2.31	28.33±2.52	25.67±1.15	20.00±3.74	20.50±4.04	24.75±3.10	**68.33±6.43**	**64.75±5.32**	47.75±5.50
L. leonurus	**99.83±0.41**	26.33±5.13	22.75±4.79	**100.00±0.00**	**55.75±3.59**	29.40±1.14	**76.40±3.58**	**67.00±3.65**	**63.80±5.36**
L. sericea	9.25±1.50	9.00±2.12	8.83±1.47	16.00±3.81	17.60±3.29	15.40±1.52	30.00±1.00	21.33±0.58	22.00±2.64
S. lancea	6.00±1.63	10.50±2.38	9.50±2.08	5.60±1.14	8.20±0.84	6.00±1.58	8.75±0.50	19.50±3.39	18.40±1.82

DCM/MeOH-dichloromethane: methanol, In bold is the good motility inhibition

The good activity shown by *C. anisata* and *L. leonurus* encouraged further testing using different concentrations for motility and J2 hatch inhibition of *M. incognita*. None of the extracts was active against the J2s, with inhibition less than 2% to 0% at 1 mg/mL (highest concentration tested) after all incubation periods being observed, except for the water extract of *L. leonurus* **(Figure 4.1)**. As shown in **Figure 4.1a**, percentage immotility with the *L. leonurus* water extract increased with time. After 48 h of exposure, 78% of non-motile juveniles was recorded at 0.7 mg/mL and 80% with 0.8 mg/mL. The good activity shown by the water extract of *L. leonurus* was supported by the lowest EC_{50} value of 0.64, 0.74 and 0.79 mg/mL at 24, 48 and 72 h respectively **(Table 4.2)**. Acetone and DCM/MeOH extracts did not show good activity, which also differs from the activity shown when testing at 1 mg/mL. The high motility inhibition after 48 h, which reduced after 72 h, was most likely due to temporary paralysis. The inactivity of *C. anisata* extracts at different concentrations disagrees with the results shown by the extracts when testing at 1 mg/mL. This could be due to the decomposition of compounds over time since the same extract was used.

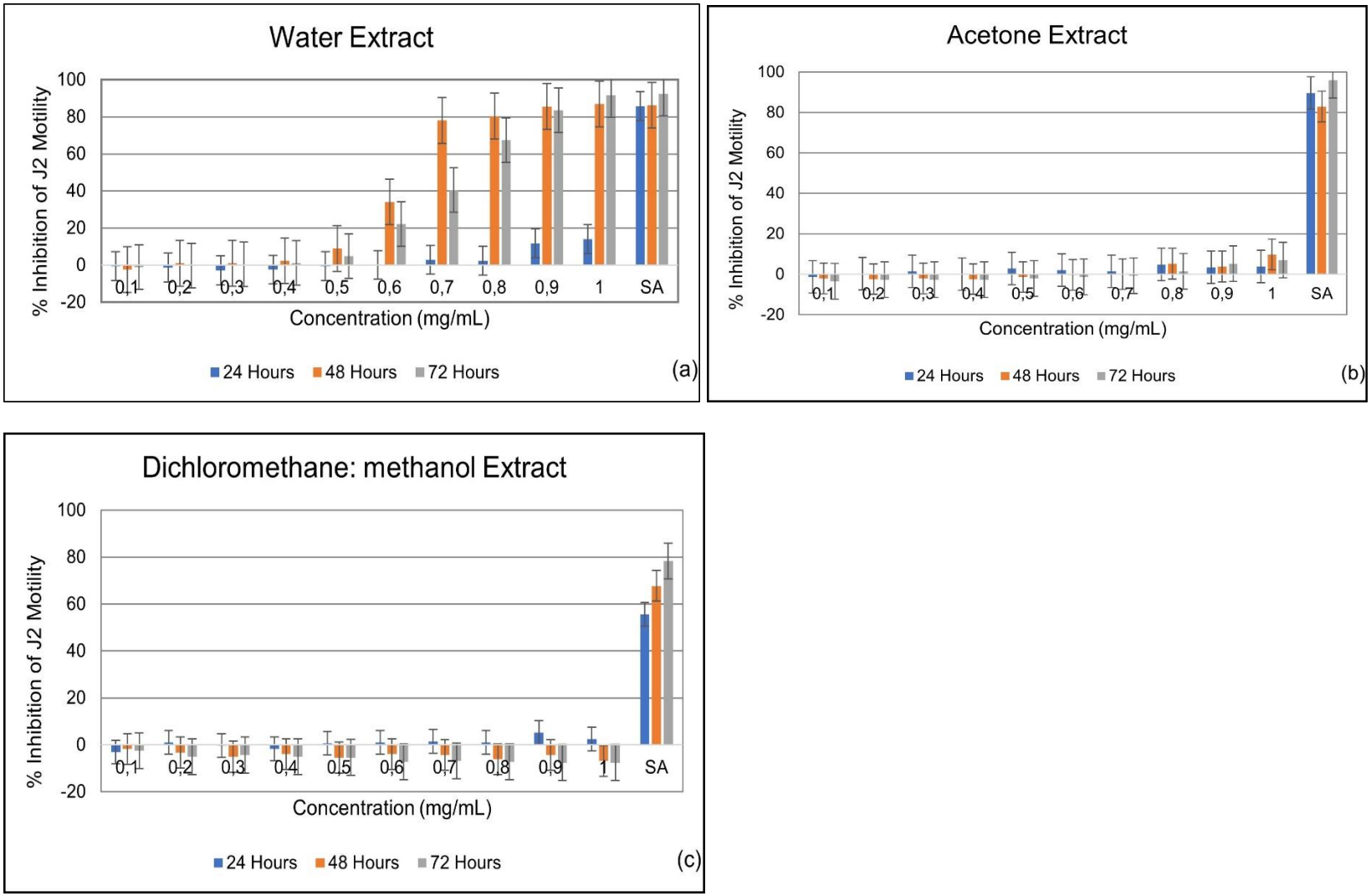
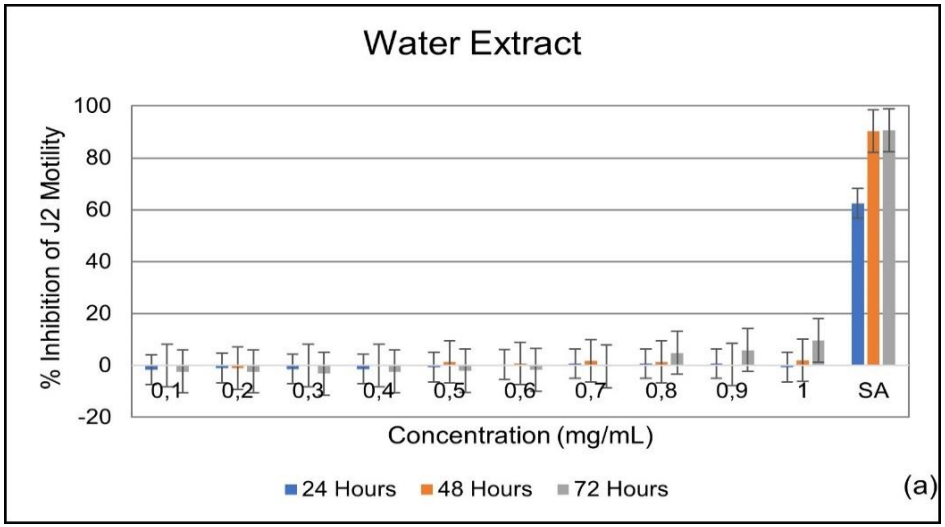
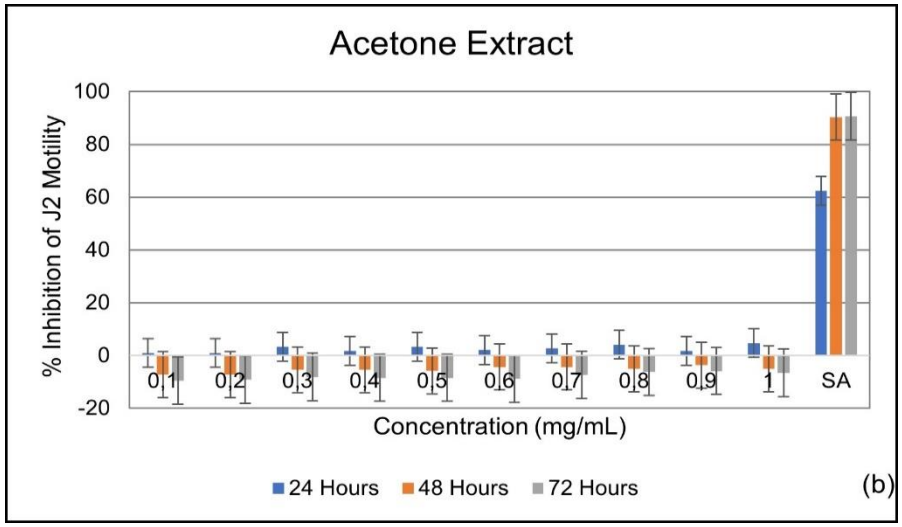


Figure 4.1: Effect of *L. leonurus* water (a), acetone (b) and DCM/MeOH (c) extracts against motility inhibition of *Meloidogyne incognita* second stage juveniles. SA – salicylic acid





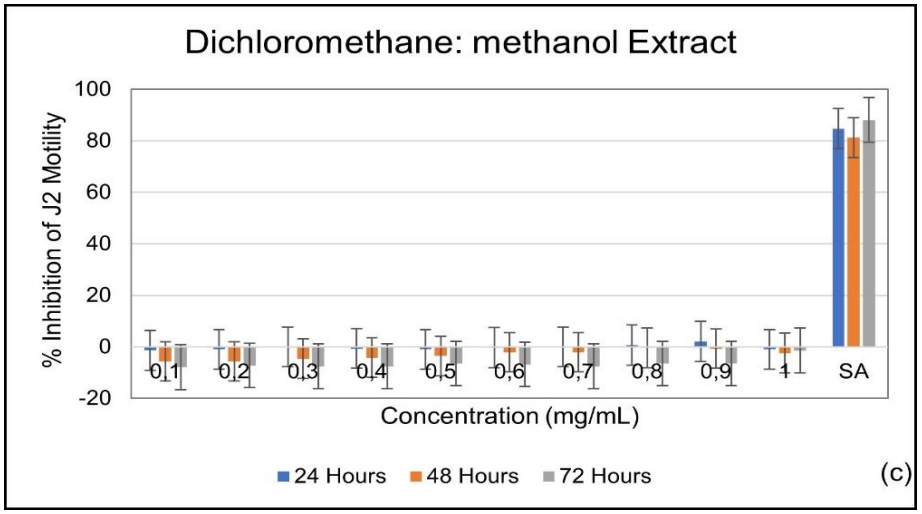


Figure 4.2: Effect of *C. anisata* water (a), acetone (b) and DCM/MeOH (c) extracts against motility inhibition of *M. incognita* second stage juveniles. SA – salicylic acid

Second-stage juvenile hatch inhibitory effects of *L. leonurus* and *C. anisata* extracts are shown in **Figures 4.3** and **4.4** respectively. According to Wuyts et al. (2006), hatch inhibition could reduce the building up of nematodes inside the roots and in the soil where plant debris is a source of active chemicals. *Leonotis leonurus* extracts were effective in inhibiting the J2 of *M. incognita* from hatching at all concentrations except for the acetone extract which had low inhibition of less than 40% even at high concentrations. Good activity was seen by the lowest EC_{50} in water (EC_{50} = 0.45 mg/mL) and DCM/MeOH (EC_{50} = 0.79 mg/mL) after 14 days of exposure **(Table 4.2)**.

Table 4.2: Effective concentration (EC_{50} in mg/mL) of *L. leonurus* and *C. anisata* extracts against *M. incognita* for juvenile motility (LM) and J2 hatch (EH) assay

Plants	Extracts	JM (mg/mL)			JH (mg/mL)		
		24 h.	48 h.	72 h.	7 days	10 days	14 days
L. leonurus	Water	4.37	0.65	0.72	0.64	0.74	0.79
	Acetone	9.45	4.70	5.24	1.53	2.37	2.04
	DCM/MeOH	8.97	15.97	11.42	1.07	0.57	0.45
C. anisata	Water	22.13	21.44	4.38	5.52	6.41	5.82
	Acetone	18.58	18.31	16.01	0.12	1.18	1.13
	DCM/MeOH	27.65	10.06	14.20	0.59	-0.03	-0.19

JM – Juvenile motility;JH – juvenile hatch

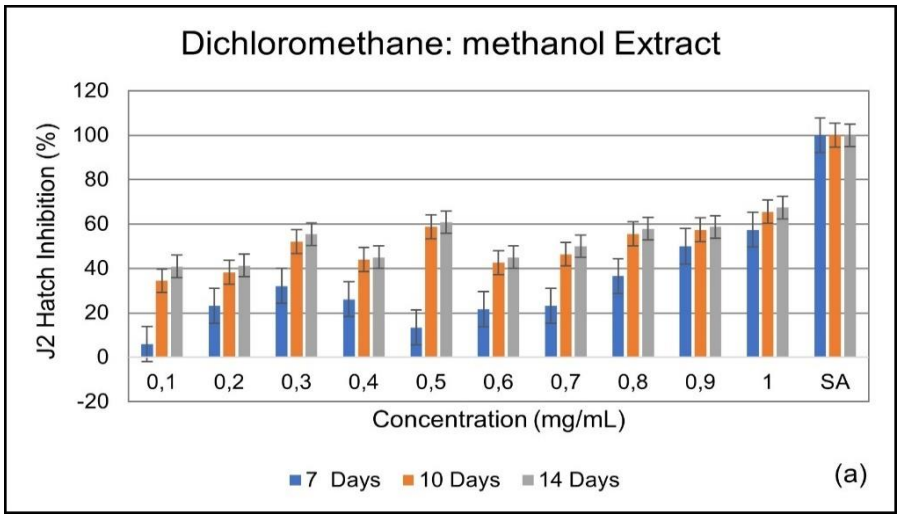
The good inhibition shown by *L. leonurus* extracts against *M. incognita* unhatched J2 could therefore assist in reducing the high population increase of nematodes in tomato roots. The activity shown by the DCM/MeOH extract in inhibiting the J2 from hatching, while not being able to inhibit motility of the second-stage juveniles, could mean that the compounds found in this extract have better inhibitory properties on the J2 hatch than direct motility effects. Wuyts and colleagues stated that concentrations applied exogenously are lower inside of eggs than outside and it is not unusual to find hatching at concentrations which inhibit nematode movement (Wuyts et al., 2006). In the present study, this was different because the J2 hatch inhibition was higher than the

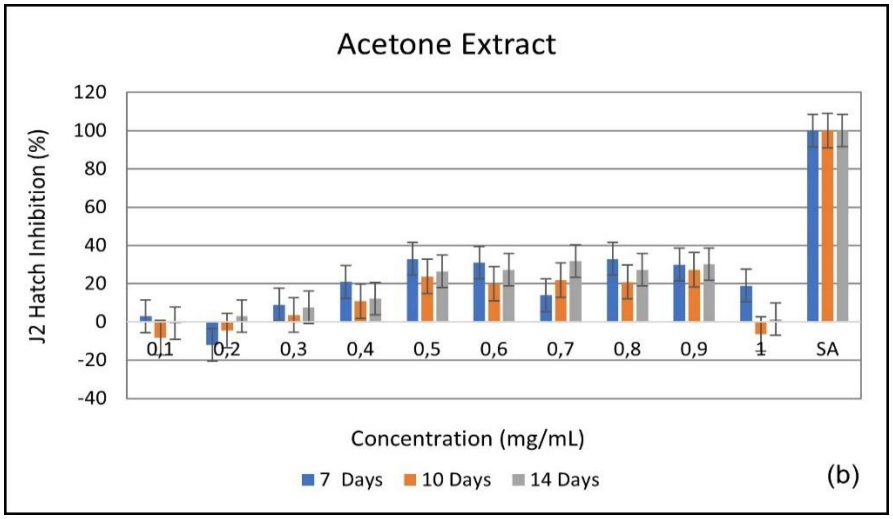
motility inhibition, implying a possible different mechanism by which the extracts might have directly destroyed the eggs and J2s respectively. The good activity shown by water extracts in both assays indicates that polar compounds in the plant are responsible for the motility inhibition and J2 inhibition, which supports traditional use. As reported by SANBI (2018) cited in Hurinanthan (2009), *L. leonurus* is traditionally used for treating intestinal worms in humans as well as in goats (Maphosa and Masika, 2010). This study is the first report of *L. leonurus* being effective against PPN. Compounds in this plant, or potentised extracts, may possibly be developed into treatments for parasitic nematodes of economic importance in agriculture as well as humans and animals. Much further work is needed in this respect.

Acetone and DCM/MeOH extracts of *C. anisata* showed good activity in inhibiting the J2 of *M. incognita* from hatching even at the lowest concentration of 0.1 mg/ml with more than 40% inhibition. The water extract had stimulating effects on *M. incognita* eggs, enhancing the hatchability of the J2. According to Hough and Thomason (1975), aldicarb has been reported to stimulate egg hatching at low concentrations and this has been attributed to the increased activity of juveniles inside eggs.

Acetone is commonly preferred as an extractant for plant material because it extracts both polar and non-polar components (Masoko and Eloff, 2006) and it has low toxicity (Eloff et al., 2007). The good activity shown by *C. anisata* acetone and DCM/MeOH extracts in inhibiting J2 from hatching is due to the presence of a number of different phytochemicals in the extracts **(Chapter 3, section 3.7.5).** The phytochemistry of *C. anisata* has been extensively studied and various compounds belonging to different chemical groups have been isolated and identified, including profiling of essential oils.

Numerous coumarins have been isolated from stem bark, fruit and aerial parts of *C. anisata*. According to Tripathi et al. (2011), coumarins have been reported to have insecticidal and repellent activity, as well as feeding deterrence, which may explain the ethnomedicinal use of *C. anisata* against pests and parasites. In African traditional medicine, the tree has been reported to treat malaria, taeniasis and schistomiasis (Hutchings et al., 1996). In the search for anthelmintic plants to test against PPN, this plant was reported to have good anthelmintic activity against *H. contortus* by Adamu et al. (2013). In their report, stronger activity was observed in the larval hatch than the larval development assay, which might explain the good inhibitory activity reported in the current study against *M. incognita* J2 hatching.





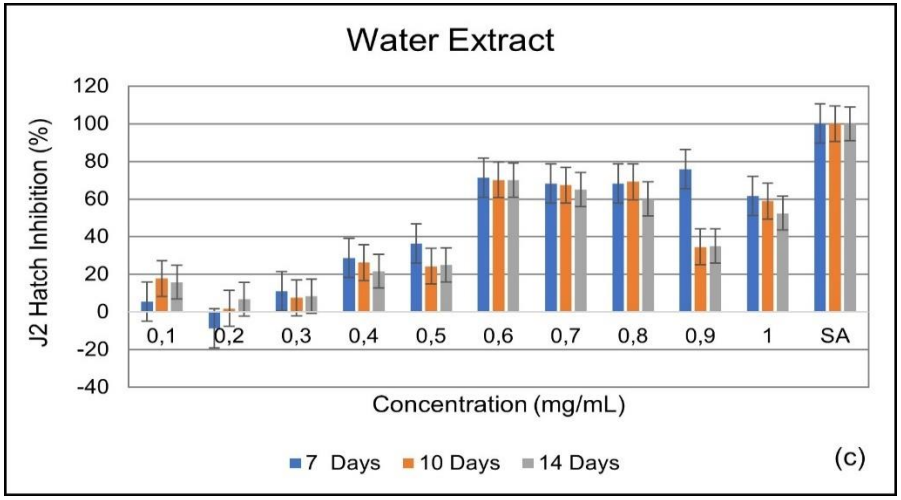


Figure 4.3: J2 hatch inhibition of *L. leonurus* (DCM/MeOH, acetone, water) extracts (a-c) exposed at 7, 10 and 14 days against *M. incognita*.

SA – salicylic acid

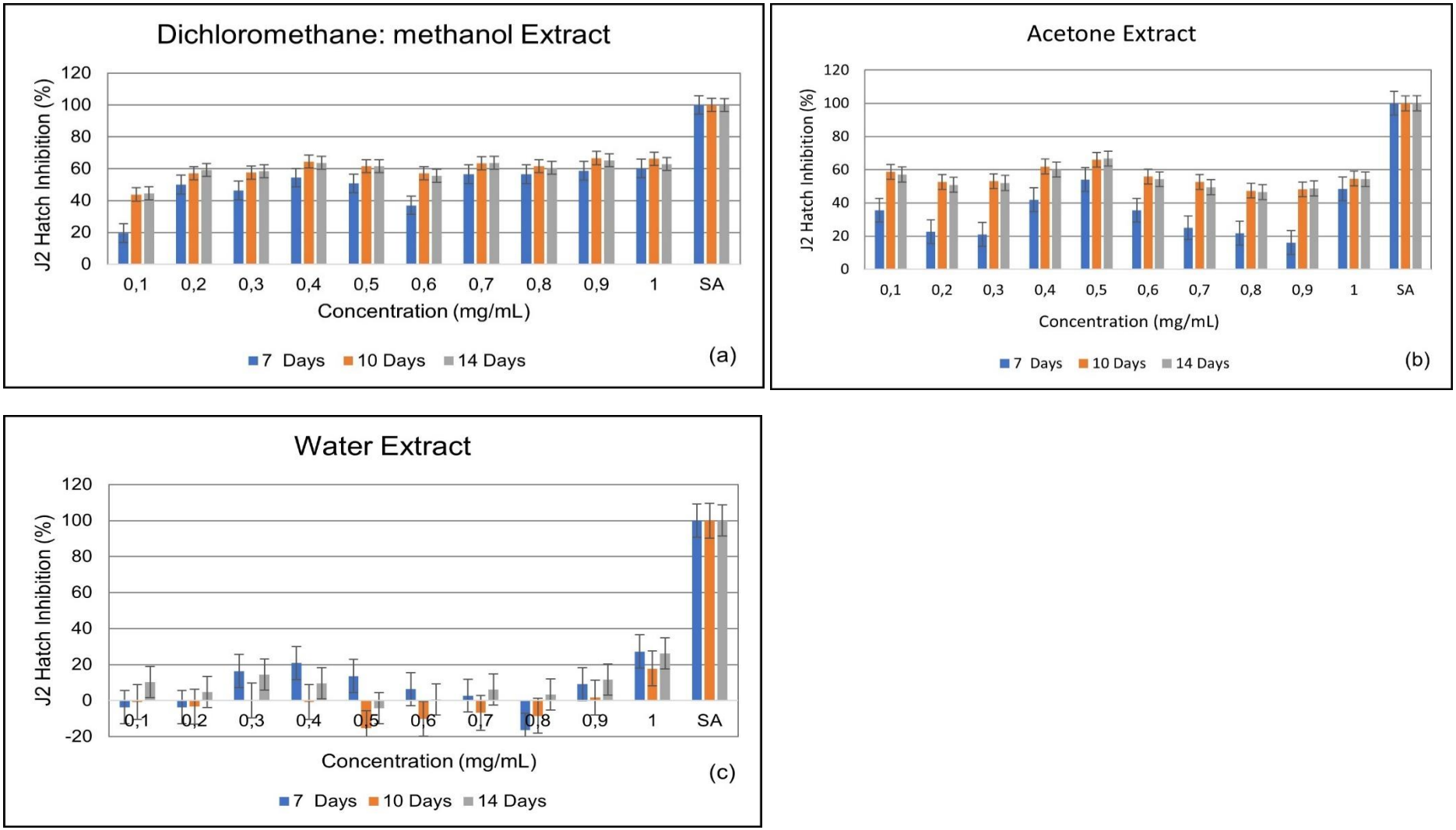


Figure 4.4: J2 hatch inhibition of *C. anisata* (DCM/MeOH, acetone, water) extracts (a-c) exposed at 7, 10 and 14 days against *M. incognita*. SA – salicylic acid

4.4.2 Antibacterial and antifungal activity

The MIC values of plant extracts against bacterial phytopathogens are presented in **Table 4.3**. According to Eloff (2004), MIC values less than 100 µg/mL are considered to be pharmacologically significant for crude extracts. A stronger and broader spectrum of antimicrobial activity was observed with the acetone extracts, followed by DCM/MeOH extracts, while those prepared using water showed weak activity with the exception of the *L. leonurus* extract which demonstrated good activity against *F. oxysporum* f. sp*. lycopersici* **(Table 4.3).** Acetone and DCM/MeOH extracts of *L. sericea* and *S. lancea* demonstrated good antibacterial activity inhibiting the growth of broad tested bacteria with MIC values ranging between 19.5 and 97.5 µg/mL. The best MIC value of 19.5 µg/mL was attained by the acetone extract of *S. lancea* against *R. solanacearum* and *X. vesicatoria,* and the *L. sericea* DCM//MeOH extract on *X. vesicatoria* as well as its acetone extract on *R. pseudosolanacearum* and *X. vesicatoria.* The second-best MIC value of 29.3 µg/mL and 39 µg/mL was observed with the DCM/MeOH extract of *L. sericea* against *R. solanacearum.*

Regarding antifungal activity, the *S. lancea* acetone extract showed moderate activity with an MIC value of 156 µg/mL in all incubation times while that of *L. sericea* was 782 µg/mL after 24 h, and further incubation resulted in MIC = 1563 µg/mL thus implying no lasting inhibition against *F. oxysporum* f. sp*. lycopersici*. The good antibacterial activity shown by *S. lancea* in the present study against tested bacterial strains is supported by other researchers working on both human and plant pathogenic microbes. Pretorius et al. (2003) reported antibacterial activity of *S. lancea* using the agar diffusion method. In their study, a mean zone inhibition of 7.0 mm against *Clavibacter michiganensis* subsp. *michiganensis* and 8.0 mm against *Pseudomonas*

solanacearum (currently known as *Ralstonia solanacearum*) was demonstrated by *S. lancea* methanolic extract. In the current study with the MIC method and different solvents used, the acetone extracts inhibited *R. solanacearum* and *C. michiganensis* subsp. *michiganensis* with MIC value of 19.5 µg/mL and 58.5 µg/mL respectively. In support of its broad antimicrobial activity, Mayekiso et al. (2009) reported the antibacterial activity of *S. lancea* on *Staphylococcus aureus, Enterococcus coli, E*. *faecalis*, *Pseudomonas aeruginosa*, *Mycobacterium smegmatis* and *M. fortuitum*. Aqueous and ethanolic stem bark extracts of *S. lancea* were also reported to be effective against *S*. *aureus, Streptococcus pyogenes, Bacillus cereus, Salmonella typhi, Shigella sonnei* and *Escherichia coli,* however there was no activity in the leaf extracts (Obi et al., 2003). According to Gundidza et al. (2008), compounds such as ∝-pinene, benzene, δ-3-carene, isopropyl toluene, and trans-caryophyllene in *S. lancea* had medicinal properties such as antibacterial, antifungal and antioxidant activities*. Leucosidea sericea* showed good activity against four phytopathogens with strong MIC values in the current study and has been previously reported to have antimicrobial activity against human pathogenic bacteria and fungi such as *S*. *aureus*, *E*. *coli, B*. *subtilis*, *S*. *sonnei*, *S*. *typhimurium*, *Klebsiella pneumoniae*, *P*. *aeruginosa*, *S*. *typhi* and *Candida albicans* (Bosman et al., 2004; Aremu et al., 2010; Pitso and Ashafa, 2015).

The best MIC value of 39 µg/mL against *F. oxysporum* f. sp*. lycopersici* was observed with the acetone extract of *C. orbiculata* followed by the water extract of *L. leonurus* with MIC value of 97.5 µg/mL, however further incubation resulted in growth of fungi with MIC of 156 and 469 µg/mL respectively **(Table 4.3)**. *Cotyledon orbiculata* leaf extract was found to have antimicrobial activity against gentamicin-methicillin-resistant

strains of *Staphylococcus aureus* (GMRSA), *Staphylococcus epidermidis*, *P. aeruginosa*, *C. albicans*, *Propionibacterium acnes* and *Microsporum canis* at MIC values varying from 0.25 to 1 mg/mL (Mabona et al., 2013). The good antimicrobial activity shown by acetone extracts in the present study could be due to the fact that acetone can extract compounds with a wide range of polarities, is volatile, miscible with water, and has low toxicity to bacteria and fungi (Eloff, 1998b).

Table 4.3: Minimum inhibitory concentration (MIC) values (µg/mL) of extracts from different plant species against test bacterial and fungal strains (mean±SD)

Plant species	Extracts	Bacterial strains					*F. oxysporum*	
		R. pseudosolanacearum	***R. solanacearum***	***C. m.* subsp. *michiganensis***	***X. perforans***	***X. vesicatoria***	**24 h**	**48 h**
L. leonurus	H_2O	469±0.22	625±0.00	938±0.44	469±0.22	469±0.22	**97.5±0.08**	469±0.22
	Ace	469±0.22	469±0.22	938±0.44	469±0.22	235±0.11	2 500±0.00	>2 500±0.00
	DCM/MeOH	469±0.22	469±0.22	938±0.44	1 875±0.88	156±0.00	782±0.66	1 250±0.00
C. anisata	H_2O	2 500±0.00	2 500±0.00	2 500±0.00	2 500±0.00	2 500±0.00	1 875±0.88	2 500±0.00
	Ace	1 563±1.33	938±0.44	1 563±1.33	625±0.00	625±0.00	1 563±1.33	625±0.00
	DCM/MeOH	1 250±0.00	2 500±0.00	2 500±00	2 500±0.00	625±0.00	2 500±0.00	>2 500±0.00
L. rugosa	H_2O	1 875±0.88	2 500±0.00	2 500±0.00	1 875±0.00	1 875±0.88	938±0.44	1 875±0.88
	Ace	937±0.88	819±1.05	469±0.22	313±0.00	156±0.00	235±0.11	469±0.22
	DCM/MeOH	625±0.00	176±0.19	469±0.22	938±0.44	156±0.00	1 563±1.32	>2 500±0.00
L. sericea	H_2O	2 500±0.00	1 875±0.88	625±0.00	625±0.00	1 562±1.33	>2 500±0.00	>2 500±0.00
	Ace	**19.5±0.00**	332±0.41	117±0.06	235±0.11	**19.5±0.00**	782±0.66	1 563±1.33
	DCM/MeOH	**39±0.00**	**29.3±0.01**	469±0.22	782±0.66	**19.5±0.00**	>2 500±0.00	>2 500±0.00
C. glabrum	H_2O	1 563±1.33	1 875±0.88	938±0.44	1 250±0.00	2 500±0.00	2 500±0.00	>2 500±0.00
	Ace	2 500±0.00	664±0.83	176±0.19	313±0.00	2 500±0.00	645±0.86	782±0.66
	DCM/MeOH	625±0.00	391±0.33	625±0.00	938±0.44	117±0.06	938±0.44	1 250±0.00
C. dentata	H_2O	2 500±0.00	>2 500±0.00	>2 500±0.00	>2 500±0.00	2 500±0.00	>2 500±0.00	>2 500±0.00
	Ace	1 250±0.00	782±0.66	469±0.22	938±0.44	1 875±0.88	1 563±1.33	1 875±0.88
	DCM/MeOH	625±0.00	625±0.00	625±0.00	1 250±0.00	703±0.77	1 250±0.00	>2 500±0.00
A. oppositifolia	H_2O	2 500±0.00	2 500±0.00	>2 500±0.00	2 500±0.00	938±0.44	1 563±1.33	1 875±0.88
	Ace	>2 500±0.00	2 500±0.00	1 562±1.33	2 500±0.00	2 500±0.00	> 2500±0.00	>2 500±0.00
	DCM/MeOH	1 250±0.00	1 562±1.33	2 500±0.00	2 500±0.00	938±0.44	1 563±1.33	>2 500±0.00
H. pauciflorus	H_2O	2 500±0.00	1 875±0.88	2 500±0.00	1 875±0.88	>2 500±0.00	1 875±0.88	2 500±0.00
	Ace	1 093±0.00	1 405±0.22	235±0.11	313±0.00	391±0.33	1 250±0.00	1 250±0.00
	DCM/MeOH	1 250±0.00	2 500±0.00	2 500±0.00	2 500±0.00	1 875±0.88	1 250±0.00	1 250±0.00

Table 4.3: MIC values (µg/mL) of extracts from different plant species against test bacterial and fungal strains (mean±SD). **Continued**

Plant species	Extracts	Bacterial strains					*F. oxysporum*	
							24 h	48 h
		R. pseudosolanacearum	***R. solanacearum***	***C. m.* subsp. *michiganensis***	***X. perforans***	***X. vesicatoria***		
S. lancea	H_2O	>2 500±0.00	>2 500±0.00	>2 500±0.00	2 500±0.00	>2 500±0.00	2 500±0.00	>2 500±0.00
	Ace	**97.5±0.00**	**19.5±0.00**	**58.5±0.03**	156±0.00	**19.5±0.00**	156±0.00	156±0.00
	DCM/MeOH	156±0.00	**48.8±0.04**	938±0.44	782±0.66	**78±0.00**	938±0.44	1 563±1.33
C. orbiculata	H_2O	>2 500±0.00	>2 500±0.00	>2 500±0.00	>2 500±0.00	>2 500±0.00	>2 500±0.00	>2 500±0.00
	Ace	332±0.41	313±0.00	313±0.00	391±0.33	469±0.22	**39±0.00**	156±0.00
	DCM/MeOH	625±0.00	1 562±1.33	2 500±0.00	2 500±0.00	781±0.66	1 250±0.00	2 500±0.00
Positive control		12±0.00	19.5±0.00	10±0.00	10±0.00	10±0.00	78±0.00	313±0.00

H_2O- Water, Ace-Acetone, DCM/MeOH-dichloromethane: methanol, in bold are the values indicating strongest inhibition

Streptomycin (2 000 µg/mL) for bacteria; Amphotericin B (10 000 µg/mL) for fungi

The most susceptible organisms in the present study were *R. pseudosolanacearum, R. solanacearum* and *X. vesicatoria. Ralstonia* and *Xanthomonas* species are associated with roots of tomatoes and since nematodes cause injury to the roots of the host plants, the resultant damage with the sign of gall formation, wilting symptoms as well as spots on leaves reduce the quality and quantity of the tomato yield (Singh and Siddiqui, 2012). The weak activity shown by *S. lancea* and *L. sericea* extracts against *F. oxysporum* f. sp*. lycopersici* is supported by the report of Heisey and Gorham (1992) cited in Pretorius et al. (2003) stipulating that plant pathogenic fungi are more resistant to natural extracts from plants than plant pathogenic bacteria. The good activity shown by plants in the present study will assist in mitigating the effects of bacterial wilt, spot, and canker as well as wilting caused by *Fusarium* species, which are caused by the investigated pathogens. A wider range of fungi affecting tomatoes will probably have to be tested to determine if *H. pauciflorus, L. sericea, A. oppositifolia, S. lancea, C. dentata* and *C. anisata* might have activity and to come to a definite conclusion regarding the antifungal status of *C. orbiculata* and *L. leonurus.*

Conclusion

The plant species selected from a literature search of plants with published anthelmintic activity against *C. elegans* or *H. contortus* had promising to good activity against motility of J2 of *M. incognita* and exhibited some degree of antibacterial effect against most plant pathogenic bacteria tested. *Leonotis leonurus* and *C. anisata* had good nematicidal activity while the acetone extracts of *S. lancea* and *L. sericea* were effective at low concentrations in most bacteria tested. Extracts of *C. orbiculata*, *S. lancea* and *L. leonurus s*howed antifungal activity. The good activity of *L. leonurus* and

C. anisata in vitro against RKN encourages further investigation regarding the isolation of nematicidal compounds as well as *in vivo* glasshouse trials to confirm the activity.

CHAPTER 5

Isolation and characterisation of nematicidal compounds from *Leonotis leonurus* acetone leaf extract

5.1 Introduction

Plant pathogens, insects and weeds are major constraints in current agricultural production, causing considerable crop losses worldwide (Malandrakis et al., 2019). Pesticides have been universally considered as the most efficient, quick, easy and inexpensive solution for controlling soilborne pathogens (Aktar et al., 2009). However, excessive and unsystematic application of agrichemical inputs, in particularly pesticides and fertilizers, have been found to possess a threat to the environment and humans which is a major problem in promoting sustainable agriculture (Zhang et al., 2018).

The undesired side effects of synthetic pesticides have necessitated the search for alternative methods for nematode control, hence the use of medicinal plants. It is important to note that not all medicinal plants are safe, concerns have been raised about the toxicity of medicinal plants. Hence it is important to screen plants for toxicity before concluding on their safety for use. Herbicides for example are effective in protecting crops as they target physiological pathways specific to plants; however, they can interfere with metabolic and reproductive processes in animals as well, often in ways that are unrelated to their specific mode of action in plants (Sánchez-Bayo, 2011). Natural product-based compounds have been reported to be non-persistent under field conditions as they are readily transformed by light, oxygen, or

microorganisms into less toxic products (Nguyen et al., 2013). Their structural complexity, involving generally higher molecular weights and greater proportions of oxygen and hydrogen allow them to be easily degraded, thus assisting in minimizing toxicity in the environment (Duke et al., 2000).

The good activity demonstrated by *Leonotis leonurus in vitro* against root-knot nematodes (RKN), free-living nematodes (FLN) and animal-parasitic nematodes has prompted further analysis on the plant to investigate the analogs that might be responsible for the activity investigated *in vitro. Leonotis leonurus* has thus far not been explored for managing PPN. The leaf water extract of *L. leonurus* showed promising activity in terms of reducing the motility of J2 of *M. incognita* in this study as shown in **Table 4.1, Figure 4.1** and **4.3**, and promising activity against juveniles of *C. elegans* **(Table 3.2)**. The plant extract was not toxic to mammalian Vero cells **(Table 3.6)** and neither were the fractions **(Table 3.7)**. The safety and the reported promising activity as potential anthelmintic plant has supported the further investigation on the plant. *Clausena anisata* also had promising activity as indicated in **Table 4.1** and **Figure 4.4** and was also fractionated to test the activity of fractions against *M. incognita*. This part of the study aimed to isolate nematicidal compounds from *L. leonurus* using *C. elegans* as a test organism in bioassay-guided isolation. *Caenorhabditis elegans* was selected for the present study as a model due to the advantages it provides in the laboratory; such as their short life cycle, small body size, high sensitivity to toxins and toxicants which enable testing various samples (Leung et al., 2008). In addition to the ease in working with this species in contrast to parasitic nematodes, is the existence of reports providing leads to development of anthelmintic

drugs through understanding their mode of action and mechanisms of resistance using *C. elegans* (Holden-Dye and Walker, 2014).

5.2 Materials and Methods

5.2.1 Plant collection and bulk extraction

The fresh leaves of *Leonotis leonurus* (L.) R. Br. were collected at the Manie van der Schijff Botanical Garden, University of Pretoria during spring (October). A voucher specimen (PRU 0125287) was deposited in the H.G.W.J. Schweickerdt Herbarium, University of Pretoria. The leaf material of *L. leonurus* was air dried at room temperature (25˚C) in a well-ventilated room and ground to a fine powder. Ground plant material (530 g) was exhaustively extracted in 9L of acetone for 6 days and filtered through Whatman No. 1 filter paper. Acetone was used for extraction since it can extract both polar and non-polar compounds (Masoko and Eloff, 2006). The filtrates were concentrated using a Büchi rotary evaporator (R-114, Labotec) at 45˚C to obtain 46.62 g of crude extract. *Clausena anisata* and *Lantana rugosa* were collected, extracted and fractionated as shown in **Section 3.2.1.1** and **3.2.1.3**.

5.2.2 Liquid/liquid fractionation

The acetone crude extract (46.62 g) was suspended in 800 mL water: acetone (90%). The dissolved extract was sequentially partitioned with *n*-hexane (3 x 900 mL), dichloromethane (3 x 900 mL), ethyl acetate (3 x 900 mL) and *n*-butanol (3 x 625 mL). All fractions obtained, including the final aqueous fractions, were concentrated. The solvent fractions (*n*-hexane, dichloromethane, ethyl acetate and *n*-butanol) were concentrated to dryness using a rotary evaporator while the water fraction was dried

in an open Petri dish under flowing cold air. All the obtained fractions were assayed for nematicidal activity against *M. incognita* and *C. elegans.*

5.2.3 Bioassay-guided isolation of bioactive compounds

The isolation of the bioactive compounds was carried out using the dichloromethane fraction, which was the most active fraction against *M. incognita* **(Figure 5.1)**. This was carried out by column chromatography using silica gel as the stationary phase. A total mass of 258.66 g of silica gel (Macherey-Nagel, Germany) with particle size 0.063-0.2 mm was packed in a dry column. Thirty (30) g of dichloromethane extract was dissolved in hexane/ethyl acetate/dichloromethane (Hex/EtOAc/DCM) (1:1:0.5) made up to 200 mL, and this was mixed with silica gel and allowed to dry under a stream of cold air. The dried dichloromethane fraction was then loaded on the column. Varying ratios of ethyl acetate and *n*-hexane of approximately 1000 mL (v/v) starting from 30% ethyl acetate and 70% *n*-hexane were used as eluent to give different fractions, gradually increasing the ethyl acetate concentration to 100%. Similar fractions based on thin layer chromatography (TLC) analysis indicating common compounds were combined to give 28 fractions. Fractions with good yield (mass above 1 g) were screened for anthelmintic activity against *Caenorhabditis elegans* following the method described in section 5.2.5 to determine the effective concentration.

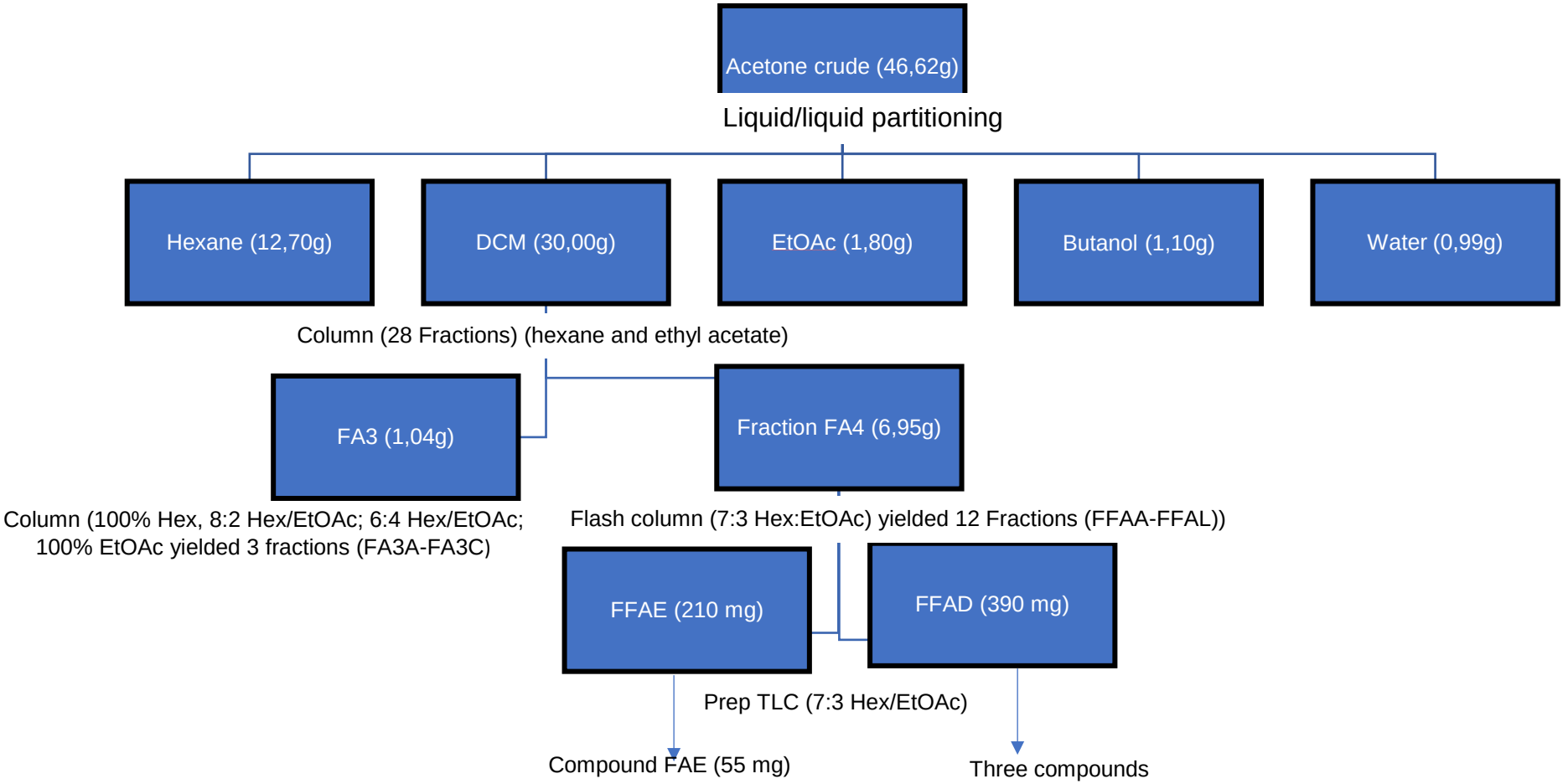


Figure 5.1: Flow diagram representing isolation of active compounds from *Leonotis leonurus*

Out of 28 fractions, fractions FA3 and FA4 had good activity against *C. elegans*. The collected fractions were analysed using TLC with Hex/EtOAc (70:30) as the eluent solvent system. Fraction FA4 (6.95 g) was further purified using flash column chromatography employing Hex/EtOAc (70:30) and subsequently 100% EtOAc which yielded 12 fractions (FFA-FFL). The FFE fraction (210 mg) was further purified using preparative TLC with Hex/EtOAc (70:30) to give compound FAE1 (Leoleorin C).

Thin layer chromatography analysis of compounds was conducted, and the plates were allowed to dry before visualisation under UV light at 254 nm. The plates were sprayed with 1% vanillin-sulphuric acid solution for visualisation. The structures of the isolated compounds were elucidated using Nuclear Magnetic Resonance (NMR) (600 and 400 MHz) spectroscopy and UPLC-QTOF-MS MS at the Department of Chemistry, University of Pretoria, South Africa. Nuclear Magnetic Resonance is a qualitative technique used for structural elucidation and to assess purity of samples (Fuloria and Fuloria, 2013). The UPLC-QTOF-MS assisted in structure elucidation by providing accurate mass and fragmentation patterns of the isolated compounds. In NMR analysis, samples were dissolved in deuterated chloroform ($CDCl_3$) while for liquid chromatography-mass spectrometry (LC–MS), UPLC acetonitrile and methanol grade were used for dissolving the compound. The 1-dimensional (1D) NMR (^{1}H and ^{13}C) spectra were analysed and compared with the literature data to conclusively confirm the structures. Liquid chromatography-mass spectrometry was used for detection of chemicals present in the extract and fractions.

5.2.4 Anthelmintic activity and cytotoxicity

5.2.4.1 Motility assay using Caenorhabditis elegans

Anthelmintic activity of fractions and compounds was determined as described in **Section 3.3.1**. Fractions were tested at concentrations of 1, 0.5 and 0.25 mg/while that of compounds at 0.5 mg/mL. The cytotoxic effects of the active fractions and the isolated compounds were investigated as described in **Section 3.4.1**. Fractions from the column where compounds were obtained, and the isolated compounds were prepared from 20 mg/mL stock solution. Six concentrations were prepared, that is 200 µg/mL, 150 µg/mL, 100 µg/mL, 75 µg/mL, 50 µg/mL and 25 µg/mL. Fractions and the compounds were redissolved in 10% DMSO to solubilise them.

5.2.5 *Meloidogyne incognita* trials

5.2.5.1 Preparation of Meloidogyne incognita inoculum

A population of *M. incognita* race 2 was prepared and cultured as described in **Section 4.2.2.1.**

5.2.5.2 M. incognita juvenile (J2) motility and J2 hatch assay

The activity of the fractions and the compounds against *M. incognita* Juvenile (J2) motility and J2 hatch assays were investigated as described in **Section 4.2.2.2.** Fractions and compounds were prepared from 10 mg/mL concentration which was redissolved in 10% DMSO for all organic solvents, while water fraction was re-dissolved in sterile water. Fractions from liquid/liquid partitioning were prepared in 10 different concentrations, 0.1 mg/mL, 0.2 mg/mL, 0.3 mg/mL, 0.4 mg/mL, 0.5 mg/mL, 0.6 mg/mL, 0.7 mg/mL, 0.8 mg/mL, 0.9 mg/mL and 1 mg/mL. Compounds and sub-fractions were prepared in 5 different concentrations, depending on the quantity some

were prepared at 0.1 mg/mL, 0.3 mg/mL, 0.5 mg/mL, 0.7 mg/mL and 1 mg/mL while others were prepared at 0.2 mg/mL, 0.4 mg/mL, 0.6 mg/mL, 0.8 mg/mL and 1 mg/mL.

5.2.6 Minimum inhibitory concentration (MIC)

Determination of the minimum inhibitory concentration of the fractions and the isolated compounds was done as described in **Section 4.2.3.2.**

5.3 Results and Discussion

Bioassay-guided fractionation of the dichloromethane fraction of *L. leonurus* led to the isolation of seven compounds. Thin layer chromatography plates showing the isolated compounds are shown in **Figure 5.2** (FA3Be, FA3Bc, FNA, FAE1, FAD1, FAD2 and FAD3).

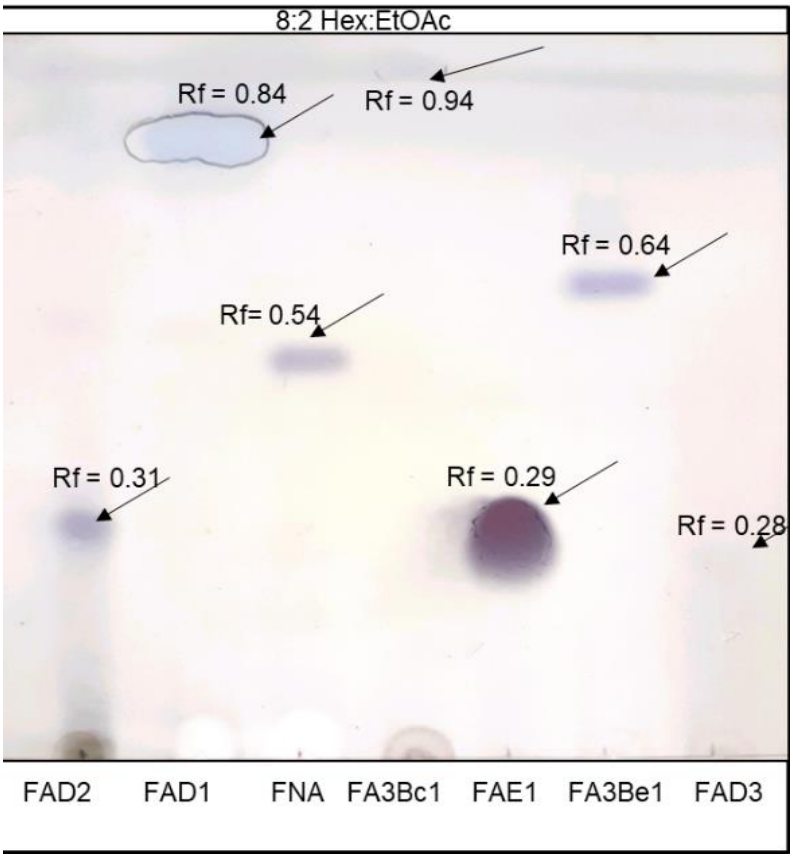


Figure 5.2: TLC plate of the isolated compounds and the retention factor (Rf) values

5.3.1 Characterisation of compound FAE1

Due to inadequate amounts of most of the compounds isolated, only Leoleorin C was fully characterised. Leoleorin C **(Figure 5.3)** was characterised by comparison with NMR data reported in the literature as recorded in **Table 5.1**. Leoleorin C (compound FAE1) was obtained as white needles and appeared as one purple spot (retention factor of 0.29) after spraying with vanillin. The NMR data was similar to that reported in the literature (Naidoo et al., 2011) **(Table 5.1)**. ^{1}H-NMR spectrum is shown in **Figure 5.4** and ^{13}C-NMR in **Figure 5.5**. The accurate mass obtained from the HR-TOF-MS spectra showed a pseudo molecular ion at *m/z* 359.2283 $[M+Na]^+$ in the ESI^+ (positive mode) which confirmed the molecular formula of $C_{20}H_{32}O_4$. Supplementary information is given in **Appendix 5.1**. Several compounds were also detected in the acetone and

dichloromethane fraction analysed with UPLC-MS. The detected compounds agree with the findings of several researchers that labdane diterpenes are highly concentrated in the leaf of the plant (Kaplan and Rivett, 1968; McKenzie et al., 2006; Naidoo et al., 2011; He et al., 2012; Wu et al., 2013). The major labdane diterpenoids were also previously identified in the acetone extract and dichloromethane fraction **(Figure 5.6)** as leolorin F, compound X, marrubiin and leoleorin B with the help of LC-MS. The isolated Leoleorin C was present in the dichloromethane fraction and the acetone extract, which helped in the identification of the compounds. Supplementary information is given in Appendix 5.2. However, these compounds share similar molecular weights with other labdane diterpenoids as follows: [leoleorin G ($C_{20}H_{28}O_4$, 332.1988), premarrubiin ($C_{20}H_{28}O_4$, 332.3392), marrubiin ($C_{20}H_{28}O_4$, 332.43392), leoleorin J (13ξ-hydroxylabd-5(6), 8(9)-dien-7-on-16, 15-olide) $C_{20}H_{28}O_4$, 332.2066)]; [compound x ($C_{20}H_{28}O_5$, 348.438), EDD ($C_{20}H_{28}O_5$, 348.2009), leoleorin J [$C_{20}H_{28}O_5$1(4α-hydroxy-9α, 13α-epoxylabd-5(6)-en-7-on-16, 15-olide), 348.2015]; [leoleorin C ($C_{20}H_{32}O_4$, 336.465680), leoleorin E ($C_{20}H_{34}O_4$, 338.48156), leoleorin F ($C_{20}H_{34}O_4$, 338.48156) and leoleorin J ($C_{20}H_{34}O_4$, 338.48156)].

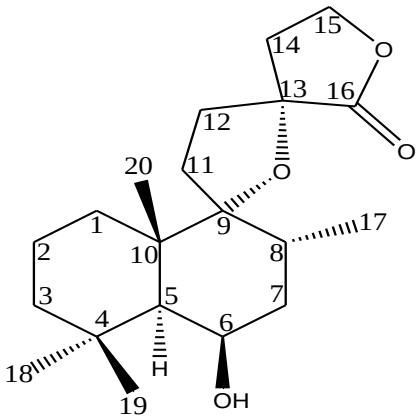


Figure 5.3: Structure of the isolated Leoleorin C (FAE1) from *Leonotis leonurus*

Table 5.1: ^{1}H and ^{13}C NMR chemical shifts of Leoleorin C (CDCl3)

	*Isolated		#Literature (Naidoo et al., 2011)	
Position	^{1}H δ(ppm)	^{13}C δ(ppm)	^{1}H δ(ppm)	^{13}Cδ(ppm)
17	0.89 (3H,d, *J*=6.8)	17.78	0.85 (3H,d, *J*=6.0)	17.8
2	1.55 (1H); 1.50 (1H)	18.7	1.55 (1H); 1.50 (1H)	18.7
20	1.27 (3H, s)	20.7	1.25 (3H, s)	20.7
19	1.21 (3H, s)	24.9	1.18 (3H, s)	24.9
11	2.32 (1H); 1.80 (1H)	29.7	2.35 (1H); 1.80 (1H)	29.6
8	2.29 (1H, d, *J*=3.0)	30.6	2.29 (1H, d, *J*=3.0)	30.6
18	0.97 (3H, s)	33.4	0.95 (3H, s)	33.3
12	2.15 (1H); 2.10 (1H)	33.5	2.15 (1H); 2.10 (1H)	33.5
1	1.58 (1H); 1.31 (1H)	34.0	1.58 (1H); 1.30 (1H)	34.0
4		34.4		34.4
14	2.41 (1H,); 2.20 (1H, m)	37.2	2.40 (1H, dd, *J* = 6.0, 13.0); 2.20 (1H, dd, *J* = 3.5, 13.0)	37.2
7	1.72 (1H, ddd, J = 2.7, 3.0, 13.6) 14.0) 1.40 (1H, ddd, J = 2.7, 3.0, 13.6)	40.8	1.70 (1H, ddd, *J* = 2.5, 3.0, 14.0) 14.0) 1.40 (1H, ddd, *J* = 2.5, 3.0,	40.7
10		43.0		43.0
3	1.26 (1H); 1.18 (1H)	44.0	1.26 (1H); 1.18 (1H, *J* = 4.5)	44.0
5	1.35 (1H, d, *J*=2.7)	49.7	1.31 (1H, d, *J*=3.0)	49.7
15	4.40 (1H) 4.30 (1H, brd)	65.4	4.38 (1H) 4.18 (1H, dd, J = 3.5, 6.0)	65.5
6	4.21 (1H, m)	68.1	4.31 (1H, ddd, J=2.5;2.5;2.5)	68.0
13		83.3		83.3
9		95.5		95.5
16		177.1		177.1

*Data obtained in $CDCl_3$, ^{13}C and ^{1}H (600 and 400 MHz); # Data obtained in $CDCl_3$, ^{13}C and ^{1}H (600 MHz

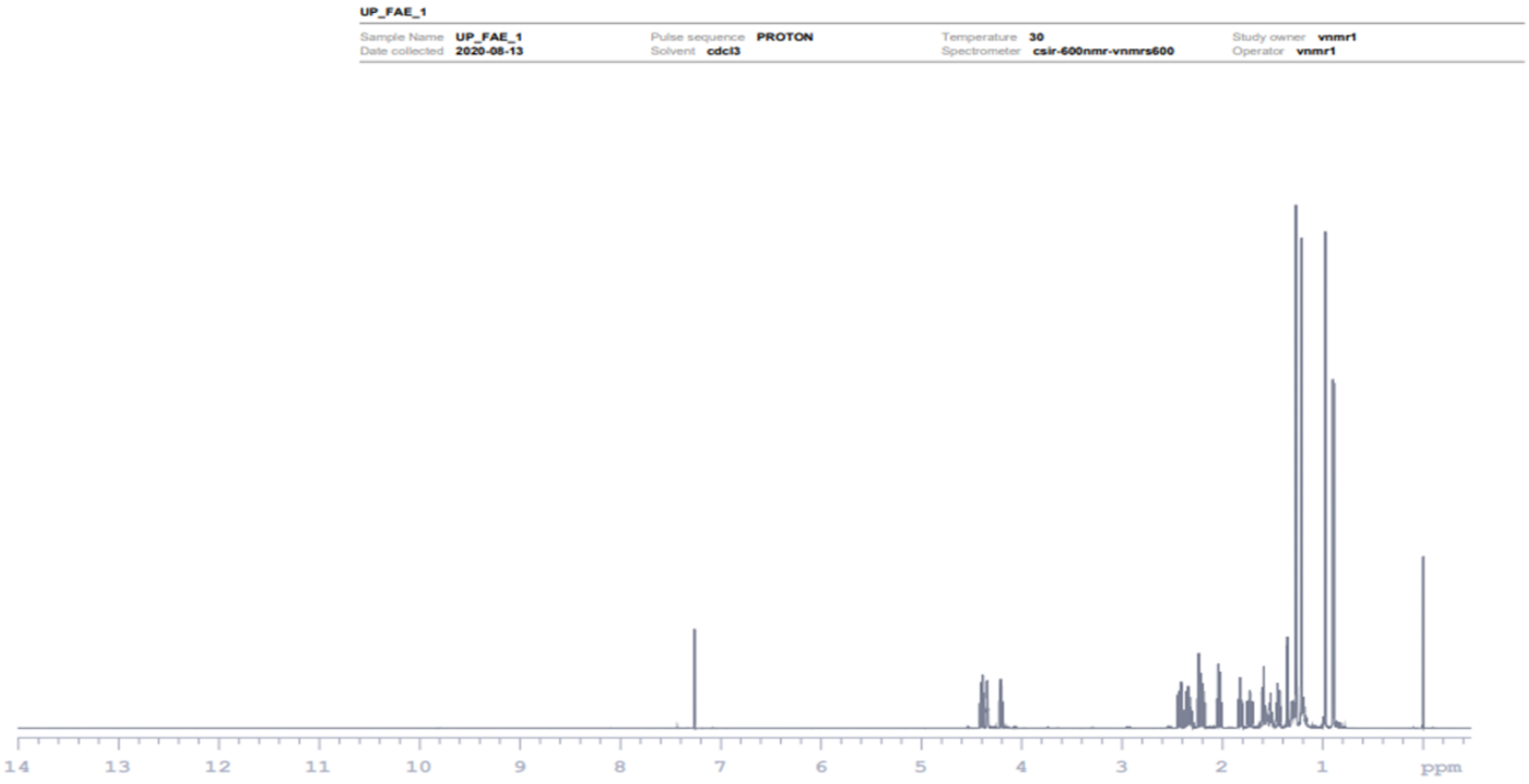


Figure 5.4: ^{1}H-NMR spectrum. Data obtained in $CDCl_3$ using 600 MHz

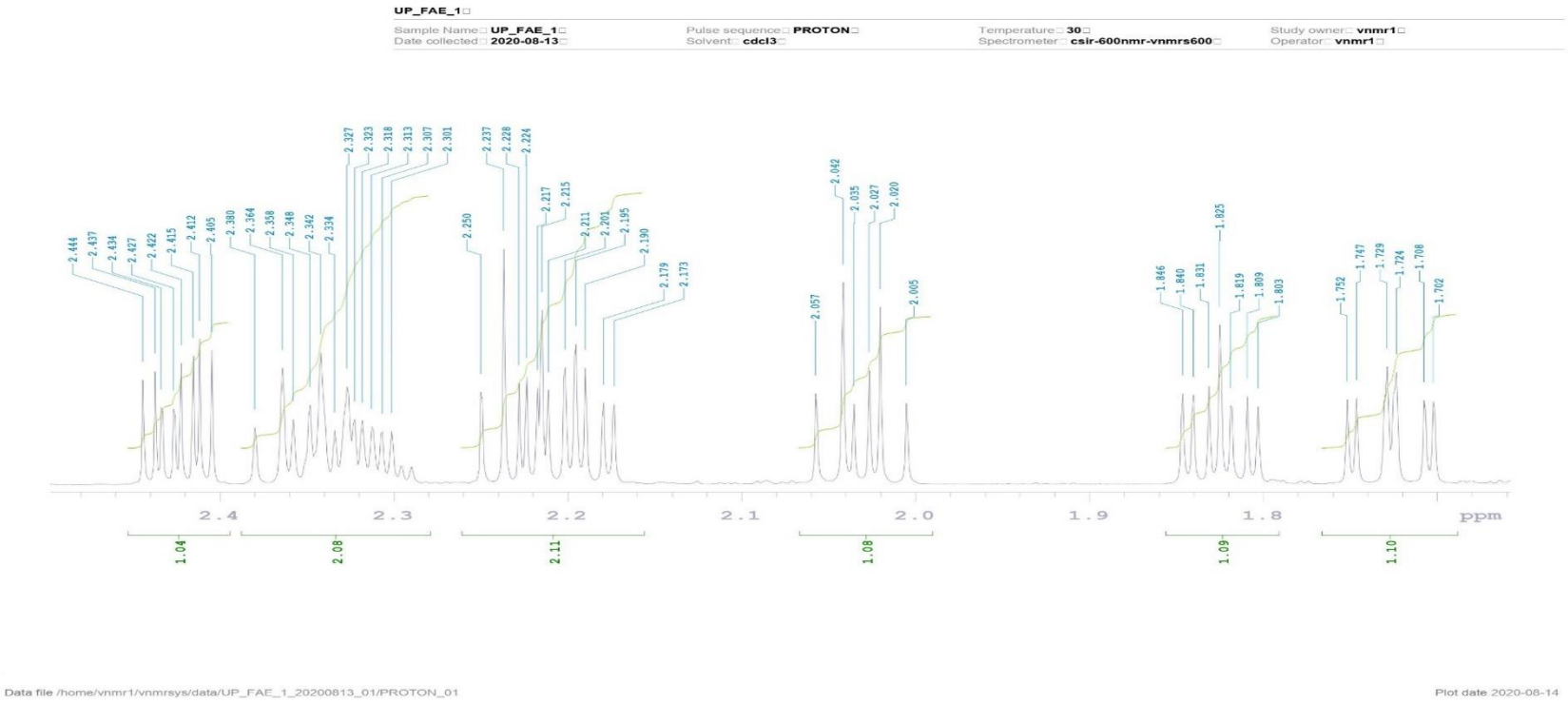


Figure 5.4: ^{1}H-NMR spectrum expansion. Data obtained in CDCl3 using 600 MHz. **Continued**

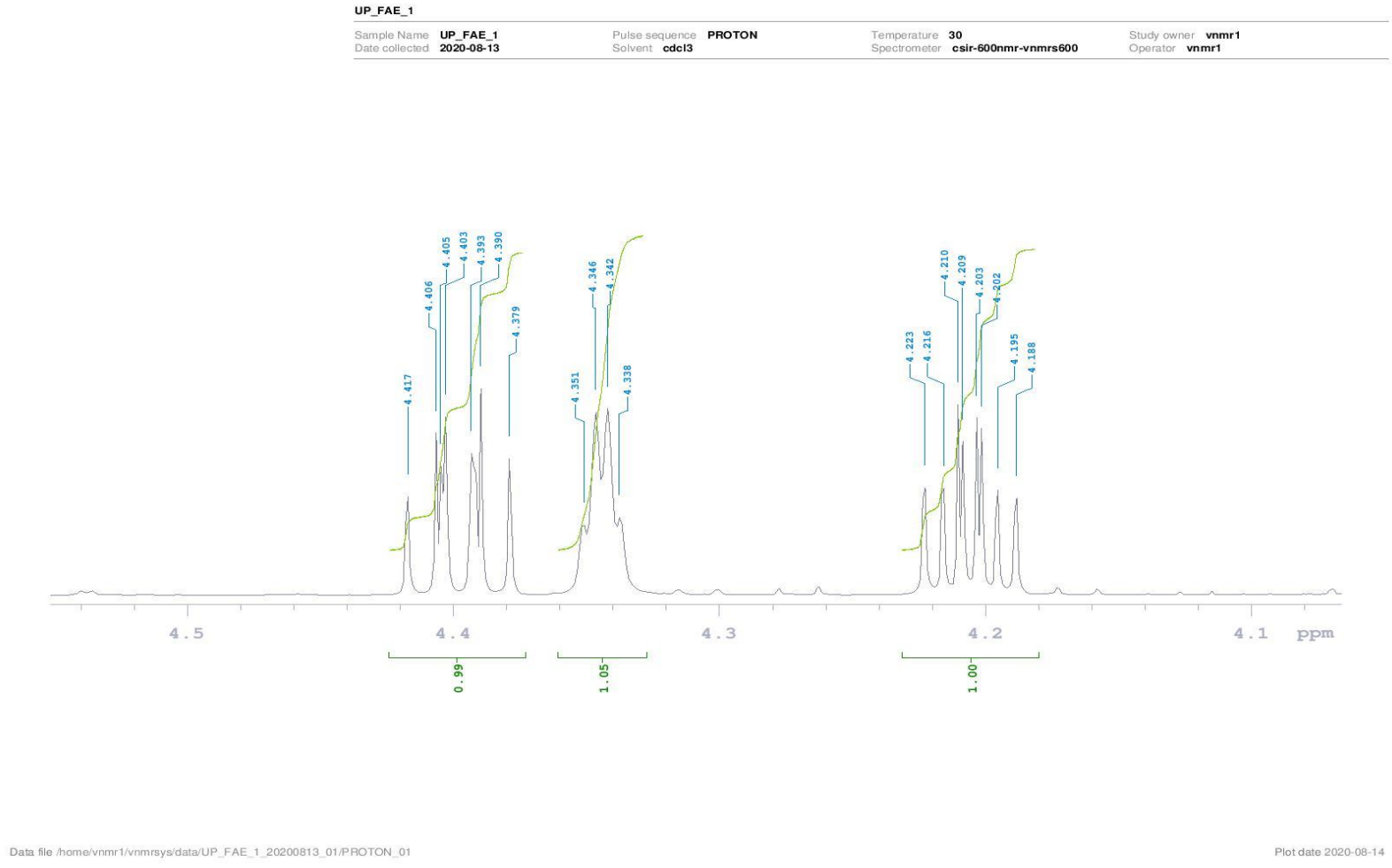


Figure 5.4: ^{1}H-NMR spectrum expansion. Data obtained in CDCl3 using 600 MHz. **Continued**

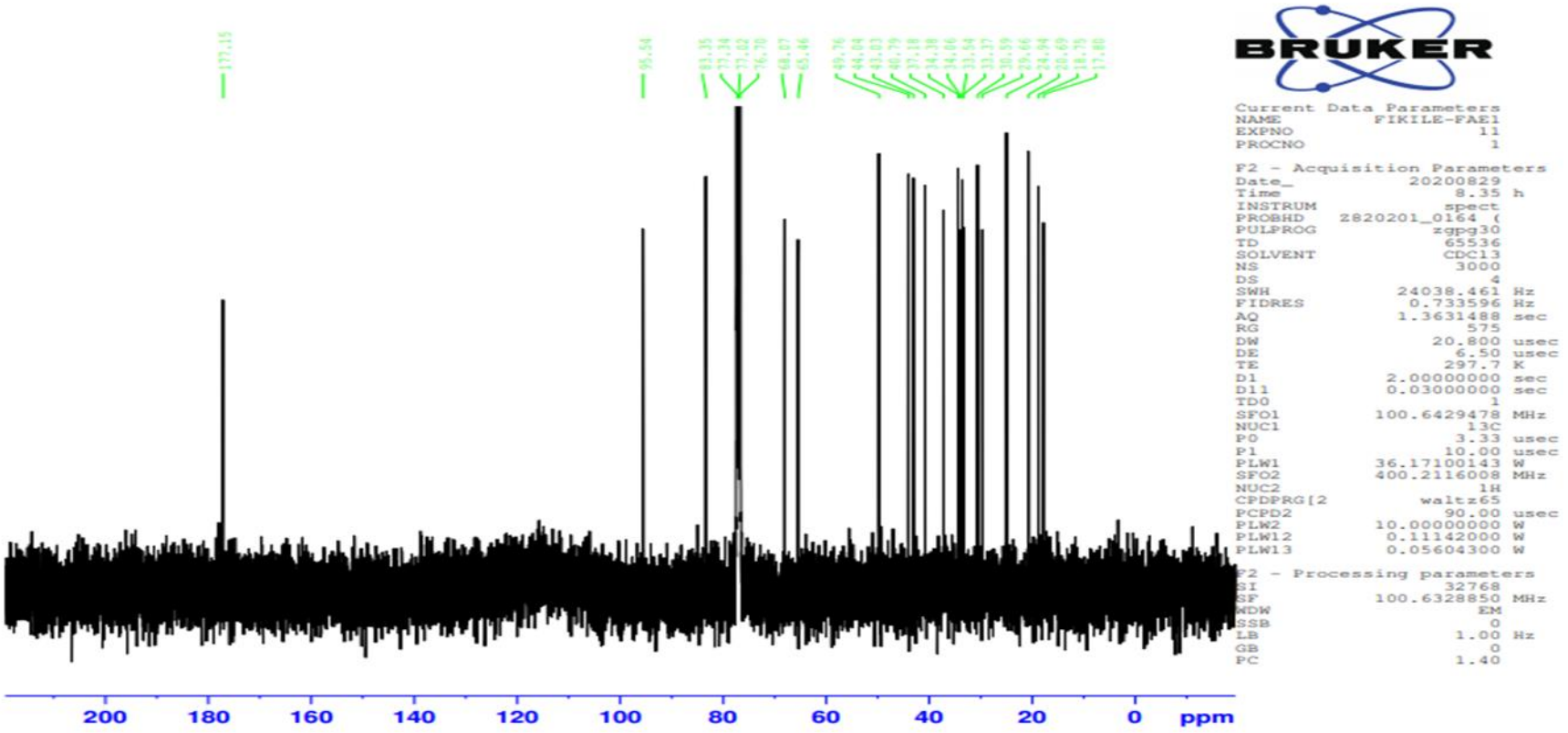


Figure 5.5: ^{13}C-NMR spectrum. Data obtained in $CDCl_3$ using and 400 MHz

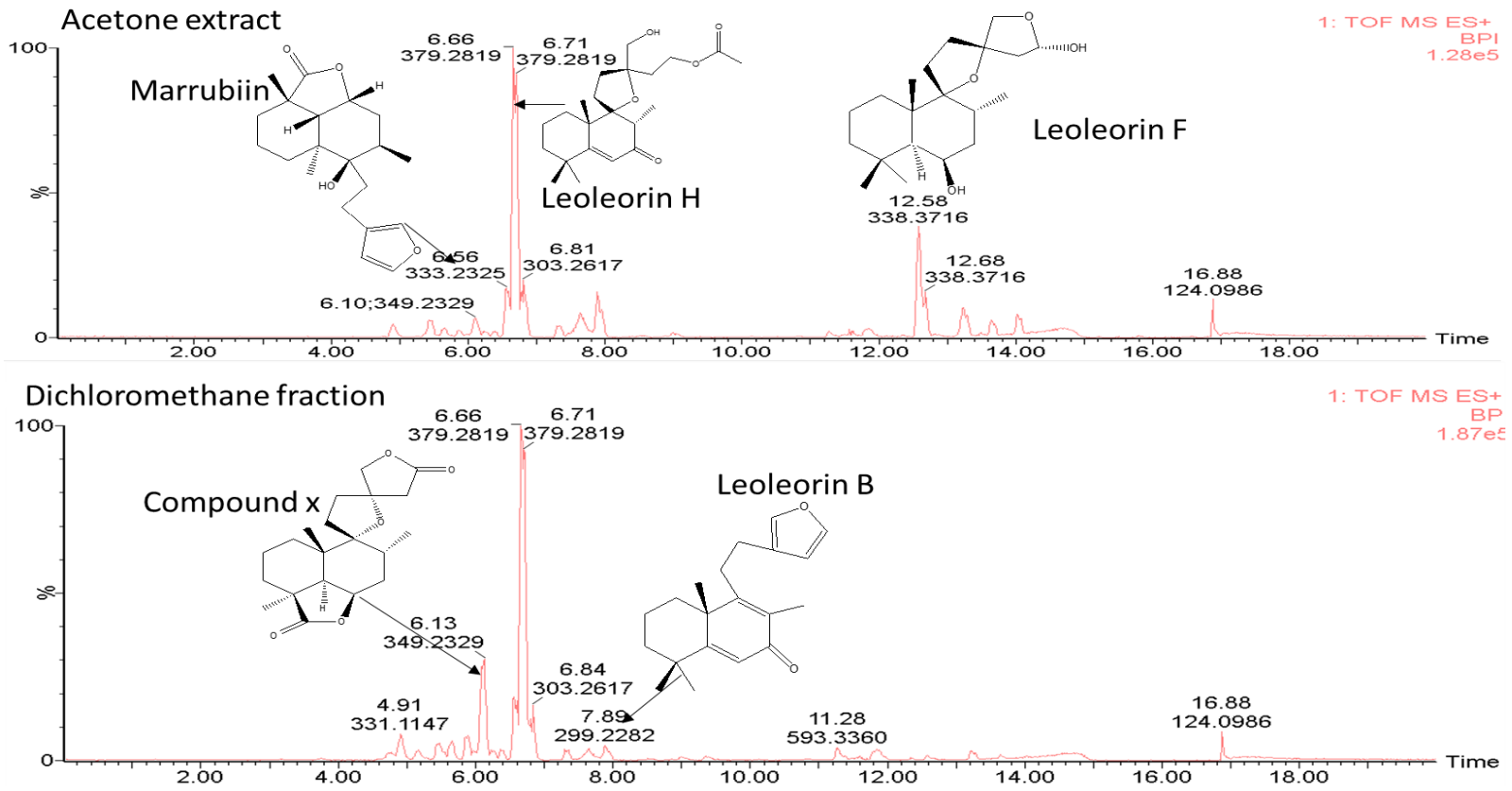


Figure 5.6: LC-MS metabolite profiles of acetone extract and dichloromethane fraction of *L. leonurus*

5.4 Anthelmintic and cytotoxic activity

The nematicidal activity of the dichloromethane fraction of *L. leonurus* against *M. incognita* prompted isolation of the constituents that might be responsible for the activity using *C. elegans* as a model. The results showing anthelmintic activity of the fractions from the column chromatography (1 and 2) of *L. leonurus* are shown in **Table 5.2**. All fractions from column 1 showed moderate to good activity. The good activity shown by Fraction FA3 resulted in it being further fractionated to get fractions as indicated in column 2. All fractions from column 2 showed good activity with motility above 80% at 0.5 mg/mL. Due to low quantity, further fractionation was done using Fraction FFAD and FFAE to isolate compounds.

Table 5.2: Fractions from chromatography columns 1 and 2 with activity against motility of *C. elegans* juveniles after incubation for 24 and 48 h

	Column 1				**Column 2**			
S/No.	Fractions	Conc. (mg/mL)	24 h	48 h	Fractions	Conc. (mg/mL)	24 h	48 h
1	F4-5	0.5	28.52	39.90	A	0.5	92.17	N/A
2		1.0	41.44	36.54		1.0	N/A	N/A
3	**F6-21 (FA3)**	**0.5**	**42.59**	**62.02**	C	0.5	69.53	84.18
4		**1.0**	**65.40**	**72.12**		0.25	36.79	53.38
5	**F22-75 (FA4)**	**0.5**	**71.53**	**N/A**		0.125	5.19	37.13
6		**1.0**	**82.21**	**N/A**	**D**	**0.5**	**59.37**	**80.38**
7	F76-131	0.5	39.16	65.38		**0.25**	**47.86**	**79.11**
8		1.0	33.46	66.83		**0.125**	**38.83**	**57.38**
9	F29-75	0.5	19.77	39.42	**E**	**0.5**	**58.24**	**91.35**
10		1.0	24.71	44.23		**0.25**	**11.96**	**56.96**
11	F175-188	0.5	52.60	54.17		**0.125**	**N/A**	**49.16**
12		1.0	41.06	52.40	F	0.5	63.43	89.45
13	F189-207	0.5	39.54	51.92		0.25	13.77	66.67
14		1.0	41.06	55.77		0.125	2.93	15.82
15	F208-226	0.5	28.14	56.25	G	0.5	68.40	85.44
16		1.0	45.63	62.02		0.25	31.38	52.53
17	F943-991	0.5	28.14	N/A		0.125	28.22	37.34
18		1.0	42.21	23.56	H	0.5	54.85	81.86
19	F125-460	0.5	36.88	28.85		0.25	52.37	60.34
20		1.0	51.71	23.56		0.125	19.86	54.22
21					I	0.5	65.69	83.54
22						0.25	64.79	77.43
23						0.125	59.59	68.78
24					J	0.5	74.94	93.67
25						0.25	77.42	84.18
26						0.125	36.79	59.49
27					L	0.5	31.61	73.63
28						0.25	N/A	48.73
29						0.125	N/A	40.51

N/A represents where the number of nematodes following incubation was higher than in the blank (% motility could not be calculated). Highlighted are the values of the active fractions used for further analysis. Strongest inhibition of juvenile motility was reflected by values greater than 71%.

Biological activity is expected to increase with further purification of plant extracts, and the isolated compounds are anticipated to display higher activity compared to the crude extracts and fractions as they would be more highly concentrated. Good activity was displayed by the sub-fractions against *C. elegans* juveniles **(Table 5.3)** compared to the dichloromethane fraction and acetone crude extract. Fractions FFAD and FFAE demonstrated good activity against *C. elegans* juveniles at the lowest concentration with motility of 80.38 and of 91.35%, respectively. Due to low quantities isolated, only four compounds were tested (Compound FNA, FA3E1, FAD1) and Leoleorin C for motility against *C. elegans* juveniles. Leoleorin C was obtained from fraction FFAE but exhibited only moderate activity. Compound FNA showed good activity and, although it was not able to be fully characterised, based on TLC and NMR data (data not shown) the compound is most likely to be a terpenoid. *Leonotis leonurus* is reported to contain mainly terpenoids (Nsuala et al., 2015) and the moderate activity shown by Leoleorin C with other compounds and good activity of compound FNA suggest that the characterised compound in the present study is not nematicidal on its own, but that compounds in the active fraction are working in synergy. All fractions, the crude extract and the isolated compound leoleorin C were relatively non-toxic to the mammalian Vero cells with all the LC_{50} values being above 0.01 mg/mL compared to doxorubicin, the positive control with an LC_{50} of 0.010 mg/mL.

Table 5.3: Cytotoxicity and anthelmintic activity of the crude extract, active fractions and isolated compounds on *C. elegans* exposed at 0.25, 0.5 and 1 mg/mL

Sample	Cytotoxicity (mg/mL)	Concentration (mg/mL)	% Motility	
			24 h	48 h
Acetone crude extract	0.072±0.006	0.5	60.36	22.17
		1.0	60.02	29.72
Dichloromethane fraction	0.020±0.000	0.5	N/A	44.0
		1.0	30.4	76.4
Fraction FA4	0.117±0.005	0.5	**71.53**	N/A
		1.0	82.21	N/A
Fraction FA3	**0.340±0.027**	0.5	42.59	62.01
		1.0	65.40	72.11
Fraction FFAD	0.169±0.016	0.5	59.37	**80.38**
		0.25	47.86	**79.11**
Fraction FFAE	0.094±0.003	0.5	58.24	**91.35**
		0.25	11.96	56.96
Leoleorin C	**0.422±0.036**	0.5	44.61	34.08
Compound FNA	**0.571±0.563**	0.5	44.78	**70.59**
Compound FA3E1	**0.422±0.036**	0.5	44.61	34.08
Compound FAD1	**0.229±0.023**	0.5	NT	NT
Levamisole		5 µg/mL	42.59	53.37
		10 µg/mL	51.71	58.65

N/A represents where the number of nematodes following incubation was higher than in the blank (% motility could not be calculated), NT denotes not tested, values in bold represent relative non-toxicity to cells in terms of cytotoxicity while in terms of motility they represent good activity. Doxorubicin was used as positive control for cytotoxicity and had LC_{50} of 0.0102±0.0025 mg/mL

5.5 *Meloidogyne incognita*

The results showing the effect of *L. leonurus* fractions against *M. incognita* J2 is shown in **Figure 5.8**. All the supporting results for *M. incognita* are shown in **Appendix 5.3**.

The water fraction had notable activity after 48 h at 0.6 mg/mL, whereas at 1 mg/mL more than 90% J2 immotility was recorded. The dichloromethane fraction showed good activity after 72 h at 0.8 mg/mL with 55% inhibition **(Figure 5.8a)**. *Clausena anisata* fractions were not active in inhibiting motility of the J2s, hence the results are not shown. The results for J2 hatch inhibition for *L. leonurus* and *C. anisata* are shown in **Figure 5.8** and **5.10** respectively. Inhibition increased with time for the dichloromethane fraction **(Figure 5.8a)** of *L. leonurus* with greater than 60% inhibition of J2 hatch after 10 and 14 days. After 7 days, some juveniles were hatching but further incubation resulted in high inhibition. Inhibition by the water fraction **(Figure 5.8b)** revealed a dose-responsive relationship with inhibition of more than 50% at 0.8 mg/mL. J2 hatch inhibition by the ethyl acetate **(Figure 5.68)** and *n*-hexane **(Figure 5.8e)** fractions was poor with average inhibition of 40% and 55% at all concentrations used and times measured. *Leonotis leonurus n*-butanol fraction stimulated J2 to hatch. In **Figure 5.10**, only the dichloromethane fraction **(Figure 5.10c)** of *C. anisata* demonstrated a dose-responsive relation in inhibiting J2 from hatching. Poor activity was also observed where inhibition was not affected by time exposure for water **(Figure 5.10a)**, *n*-butanol **(Figure 5.10b)**, and *n*-hexane **(Figure 5.10d)** of *C. anisata*. The ethyl acetate fraction **(Figure 5.10e)** was active, but the activity was only influenced by time with different concentrations yielding the same effect.

All the subfractions and the isolated compounds were not active in inhibiting the motility of J2s **(Figure 5.11 and 5.12)** with the exception of fraction FA4 which demonstrated moderate activity at all tested concentrations (0.2 mg/mL to 1 mg/mL) **(Figure 5.11)**. The isolated and characterised leoleorin C (FAE1) was unfortunately

not active in inhibiting the motility of J2s (Figure 5.13a) and had stimulatory effects **(Figure 5.13b)**.

5.6 Antimicrobial activity

Minimum inhibitory concentration results for the crude extract and the fractions of *L. leonurus* are shown in **Table 5.4**. Minimum inhibitory concentration values less than 100 µg/mL are considered to be pharmacologically significant for crude extracts and fractions (Eloff, 2004). Due to the low quantity of the isolated compounds, MIC values could not be determined, but bioautography was used as a method for evaluating the presence of antibacterial activity.

Fractions and the acetone extract had moderate to poor antibacterial activity with MIC values ranging from 117-2 500 µg/mL against all the tested bacterial phytopathogens **(Table 5.4)**. Bioautography of the active fractions and compounds on the selected bacterial pathogens were evaluated on TLC plates employing Hex: EtOAc (70:30) as mobile phase **(Figure 5.7)**. In contrast, all fractions and the crude extract showed good activity against all the phytopathogens when screened using TLC (results not shown). Fraction FA4 had moderate activity with MIC of 117 µg/mL against *Cmm* and good activity was observed in bioautography where different compounds showed inhibition. Leoleorin C did not inhibit the growth of all the tested bacteria when evaluated using bioautography except for *Cmm* where it showed promising activity **(Figure 5.7)**. The inactivity of the compound against the selected phytopathogens was also observed by Naidoo et al. (2011) against *Mycobacterium tuberculosis*. *Clavibacter m. subsp. michiganensis* causes bacterial wilt and canker in tomato and is considered to be one of the most important bacterial disease of tomatoes (Eichenlaub et al., 2007). Inhibition

of compounds against this bacterium and other phytopathogens will assist in developing broad-spectrum antimicrobial agents for diseases infecting crops, particularly tomato.

Table 5.4: Minimum inhibitory concentration (MIC) values (µg/mL) of the acetone extract and fractions against test bacterial and fungal strains (mean±SD)

Fractions	**Bacterial isolates**				
	R. pseudosolanacearum	***R. solanacearum***	***C. m. michiganensis***	***X. perforans***	***X. vesicatoria***
Acetone extract	469±0.22	469±0.22	938±0.44	469±0.22	235±0.11
n-Hexane	2 500±0.00	1 250±0.00	1 250±0.00	>2 500±0.00	1 407±1.55
n-Butanol	>2 500±0.00	2 500±0.00	1 563±1.33	2 500±0.00	>2 500±0.00
Ethyl acetate	469±0.22	313±0.00	313±0.00	625±0.00	469±0.22
Dichloromethane	313±0.00	469±0.22	391±0.33	625±0.00	313±0.00
Water	>2 500±0.00	2 500±0.00	1 250±0.00	2 500±0.00	>2 500±0.00
Fraction FA4	>2 500±0.00	313±0.00	117±0.06	2 500±0.00	1 875±0.88
Streptomycin	12±0.00	19.5±0.00	10±0.00	10±0.00	10±0.00

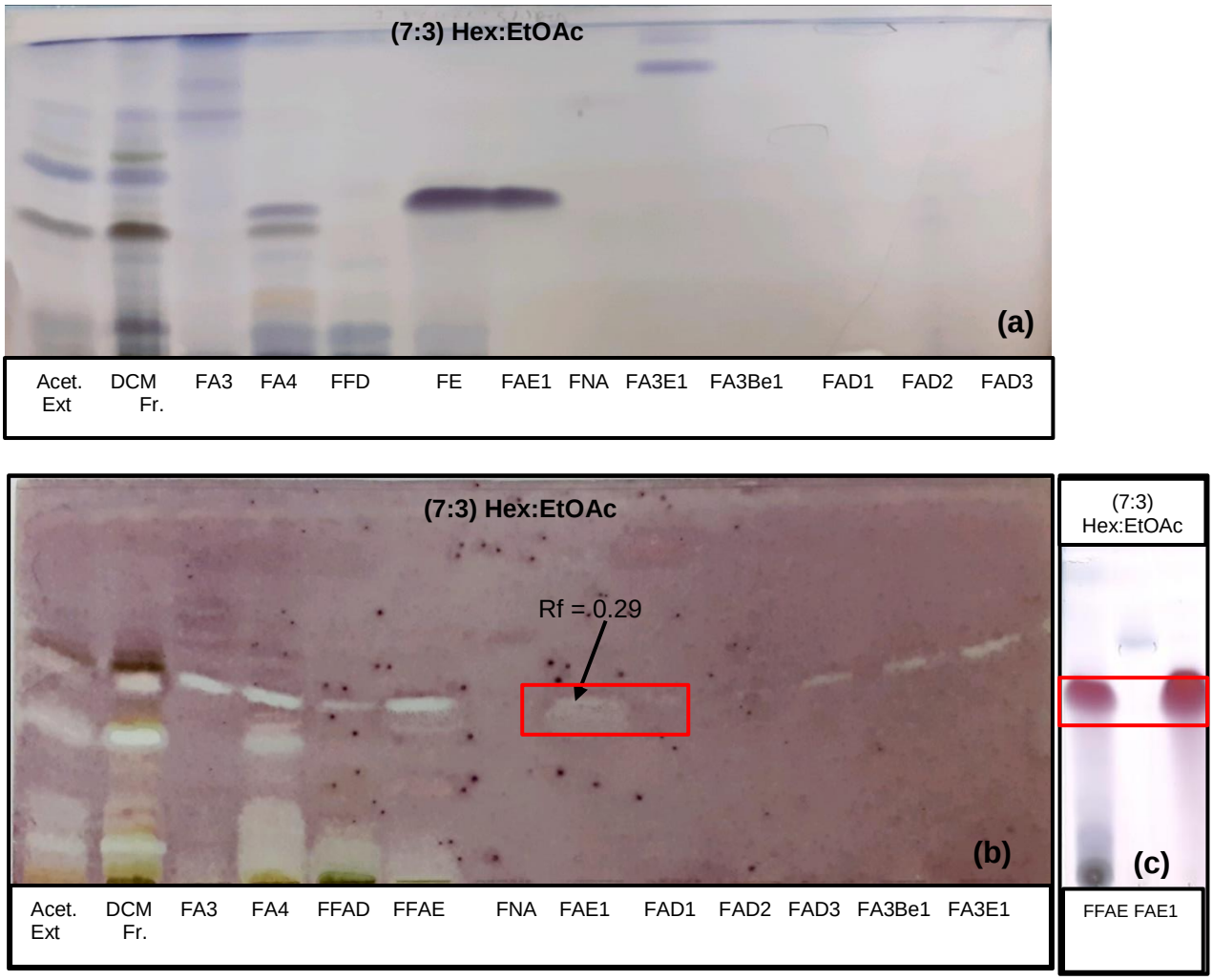


Figure 5.7: Fractions and the isolated compounds from *L. leonurus* eluted in 7:3 Hex:EtOAc. TLC plate sprayed with vanillin (a) and bioautogram sprayed with *Cmm* (b)

Highlighted in the red box is the compound leoleorin C (FAE1) sprayed with *Cmm* showing inhibition (b), and TLC plate sprayed with vanillin showing the fraction FFAE where the isolated leoleorin C was obtained (c).

Conclusion

Bioassay-guided fractionation of the dichloromethane fraction led to isolation of leoleorin C. This compound has previously been isolated from *L. leonurus*, but activity against J2 motility and J2 hatch inhibition of *M. incognita*, motility against *C. elegans* juveniles, selected phytopathogens and cytotoxicity against Vero cells is reported for

the first time in this study. Fractions showed good activity against C. *elegans,* but only moderate activity against *M. incognita*. Leoleorin C also had moderate activity against *C. elegans* but weak activity against *M. incognita*. Thus, activity against the free-living *C. elegans* could not translate well to activity against PPN with both the compounds and the fractions. The fractions and the acetone crude extract had moderate to weak activity against all the tested bacterial phytopathogens in the broth microdilution assay, but good activity was attained in bioautography. Leoleorin C was not active against any of the tested phytopathogens but had promising activity against *Cmm*. It is recommended that the dichloromethane fraction and fraction FA4 should be further explored and the bioactive compounds responsible for nematicidal activity be isolated using the nematode of interest. Lastly, the crude extract of *L. leonurus*, fractions and the isolated compounds may possibly be useful in developing alternatives for controlling both nematodes and phytopathogens of important crop species such as tomatoes.

CHAPTER 6

Efficacy of *Leonotis leonurus*, *Clausena anisata* and *Lantana rugosa* on growth of tomato seedlings and suppression of *Meloidogyne incognita in vivo*

6.1 Introduction

Root-knot nematodes (RKN) damage plants by weakening the root tips, thus resulting in the formation of giant cells on the infected root tip (Nguyen et al., 2018). Above-ground symptoms generally include stunting, leaf chlorosis and patchy growth (Nicol et al., 2011), and these are the most common symptoms which often resemble those associated with nutrient deficiencies (Osei et al., 2011), and hence are usually not linked to RKN infection. The damage caused to the plant is due to the diverted nutrients used by the nematodes over several weeks for their life-cycle completion (Caromel and Gebhardt, 2011). Chemical control is the most effective and widely used nematode control strategy. However, the adverse effect of toxic nematicides on animals, humans and the environment and their high prices render them impossible and impractical for the smallholding farming sector. Hence alternative control measures for nematodes are required.

Plants are known to be a good source of new pesticide molecules (Oka, 2012) due to the presence of secondary metabolites. They can potentially meet the increasing demand for environmentally friendly nematicides (Oka, 2012). Allelochemicals, as

reported by Kokalis-Burelle and Rodríguez-Kàbana (2006), are biopesticides which are released into the environment through volatilization, exudation, leaching (either from the plant or plant residues), or through decomposition of the residues. There are, however, negative effects associated with allelochemicals on plants, such as germination inhibition (Mafeo et al., 2011) and the suppression of seedling growth (Bhatt and Todaria, 1990). Plants could offer alternative control measures; however, their toxicity cannot be ignored so it is important to test phytotoxicity.

This aspect of the study was initiated following the good *in vitro* activity against *Meloidogyne incognita* second-stage juvenile (J2) motility and hatching detected in the extracts of *L. leonurus*, *C. anisata* and *L. rugosa* as shown in **Chapter 4**, **Table 4.1**. The activity of these plants prompted further analysis on their effect on the germination of tomato seedlings (*in vitro*) in the following section. Several compounds belonging to different classes have been isolated from *C. anisata* and *L. leonurus*, with some reported to have pesticidal activity. The successful use of allelochemicals in managing plant-parasitic nematodes (PPN) relies on the degree of non-phytotoxicity to the protected crop. *Clausena anisata* and *L. leonurus* were further analysed for their activity against *M. incognita* under glasshouse conditions.

6.2 Materials and Methods

6.2.1 Plant collection and extraction

The fresh leaves from *Leonotis leonurus* (L.) R. BR (PRU 0125284) and *Clausena anisata* (Willd.) Hook.f. ex Benth (PRU 0125285) were collected at the Walter Sisulu Botanical Gardens, Ruimsig, Johannesburg, Gauteng, SA in the spring months.

Lantana rugosa Thunb. (PRU 124386) was collected at the University of Pretoria experimental farm. Voucher specimens (PRU) were prepared and deposited in the H.G.W.J. Schweickerdt Herbarium, University of Pretoria, Tshwane, Gauteng, SA.

6.2.2 Plant preparation and extraction

The collected leaf material was dried at room temperature in a well-ventilated room and ground to a fine powder in a Macsalab Mill (Model 2000 LAB Eriez). *Leonotis leonorus*, *C. anisata* and *L. rugosa* were extracted in water as described in **Section 3.2.1.2.** Plants were extracted in water as universal solvent to mimic the natural environments when used as soil amendments, and to reduce potential toxicity on the tomato seedlings that might be brought up by organic solvents.

6.2.3 Seed germination

The study was conducted at the University of Pretoria, Plant Sciences Complex in a growth chamber (16-h light/8-h dark regime) (Phytotron 1) in October 2019, and the experiment was repeated in October 2020.

Analysis of the effect of plant extracts on the germination of tomato seeds was carried out according to the method described by Hernández-Herrera et al. (2014). For each treatment, 10 seeds of tomato were used. Experimental units were arranged in a RCBD. Seeds were surface sterilized in 4% sodium hypochlorite solution for 10 min and subsequently triple-rinsed in sterile distilled water. Tomato seeds were placed on Whatman No 5 filter paper in sterile 90 mm Petri dishes and then treated with 5 mL

distilled water (control) and of 3 different concentrations of selected water extracts (0.2, 0.4 and 10 mg/mL) **(Figure 6.1a)**. Each treatment has its own control. The different concentrations were prepared from 100 mg/mL stock solution obtained by dissolving the extracts in distilled water. The plates were incubated at 25±1˚C and a 16-h light/8-h dark regime. Germination was considered to have happened once the radicle protruded more than 2 mm. After 12 days, the effects of extracts on the growth of tomato seedlings were measured with a vernier caliper **(Figure 6.1b)**. Variables measured included plumule length, radicle length, total plant height and dry mass of tomato seedlings. Dry mass was obtained with an electronic balance after oven-drying at 60°C. Germination percentage was calculated on the final day of treatment following the calculation described by Al-Mudaris (1998):

Germination percentage % = (Total number of germinated seeds)/(Total number of sowed seed) X 100%

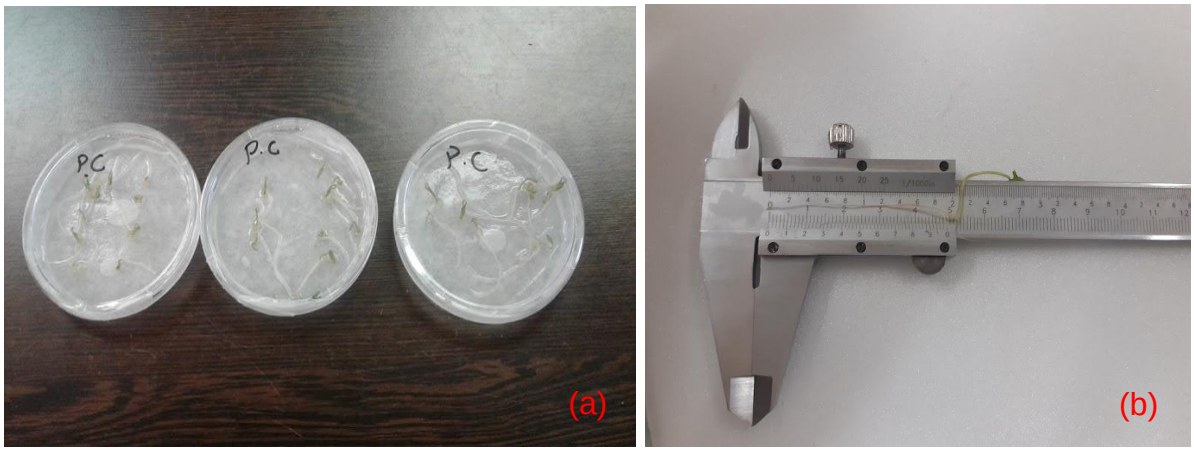


Figure 6.1: Plates of seedlings treated with extracts (a) and tomato seedling measured (mm) using a vernier caliper (Photos by FN Makhubu)

6.2.4 Glasshouse trials

6.2.4.1 Plant growth conditions

The study was conducted under glasshouse conditions, with the first experiment being conducted at the ARC-Vegetable and Ornamental Plants Campus (ARC-VOPI), Roodeplaat, Tshwane, Gauteng, SA during spring (August-October 2019). The second experiment was repeated during spring (September-December 2020) at the University of Mpumalanga, Mbombela, Mpumalanga Province, SA.

Figure 6.2: Glasshouse trial layout in Roodeplaat, ARC-VOP, Tshwane, Gauteng (Photo taken by FN Makhubu)

The glasshouse trial for evaluating nematicidal potential of powdered material of *L. leonurus* and *C. anisata* was conducted following the method described by Taylor and Sasser (1978). The soil to be used in the glasshouse trial was steam-pasteurized sandy loam soil (84% sand, 14% loam, 2% clay). Two 6-week-old seedlings of tomato

var. Rodade were transplanted in each of the (60) 5L plastic pots. Immediately after transplanting, all seedlings were inoculated with ± 3 000 eggs and J2 of *M. incognita* in 5-cm deep holes around the seedling using a 20 mL plastic syringe. The trial consisted of treatments (*L. leonurus* and *C. anisata*), control (Rugby® 100 G Nematicide, an anticholinesterase compound) and reference plant material (*Maerua angolensis*) and tested at 5, 10 or 15 g of plant material per unit of soil. *Maerua angolensis* was used as reference since it has previously been reported to reduce the population density of *M. incognita* in glasshouse, microplot and field conditions trials (Khosa, 2013; Khosa et al., 2020). The soil amendments were applied on the same day as the nematode inoculation but in a separate 5 cm deep hole. Treatments were replicated 4 times and the trial was arranged in a RCBD. During the duration of the trial, irrigation was applied by pouring ± 300 mL tap water every second day into each pot.

The trial was terminated 65 days after transplanting of the seedlings. The stem height, fresh shoot mass and dried shoot mass were recorded. Dried shoot mass was obtained after drying at 65℃ The root systems were removed from the soil and cleaned in water to measure the fresh root mass. Root samples were collected to quantify the number of nematode eggs and J2. The root systems were cut into pieces and extracted with 1% NaOCl solution (Hussey and Barker, 1973). The extracted J2 larvae and eggs were made up to 100 mL with distilled water and stored in a fridge at ± 8°C and were counted using a compound microscope (10× 40 magnification).

To determine the level of infestation, a nematode rating chart was used as described by Bridge and Page (1980) on a class of 0 to 10. The classes were described as

follows: 0 = no galling, 1 = few small knots that are difficult to find, 2 = clearly visible small knots, 3 = some large knots visible, 4 = large knots predominant, 5 = 50% of the roots infested with knotting on parts of the main roots, 6 = visible knotting on the main roots, 7 = majority of main roots are knotted, 8 = all the main roots are knotted, 9 = severe knotting on the main roots and 10 = all roots severely knotted, plants usually dead.

6.3 Statistical analysis

The data from each trial were subjected to analysis of variance (ANOVA) using Statistix 10 (Statistix 10, copyright © 1985-2013, analytical Software). The standardized residuals of each variable were tested for deviations from normality using Shapiro−Wilk's test. Discrete data were transformed through log10(x + 1) to homogenise the variances (Gomez and Gomez, 1984) but untransformed means were reported.

6.4 Results

6.4.1 Seedling germination

There was a statistically significant difference ($p<0.05$) between trial 1 and 2 for the seed germination, hence data was presented separately **(Appendix 6.1)**. Only the dry mass between the two trials were not significantly different ($p>0.05$). The effect of water extracts of *C. anisata*, *L. leonurus* and *L. rugosa* on the growth of tomato seedlings is shown in **Table 6.1** and **6.2** for trial 1 and 2, respectively. The results showed that the effect of the extracts on the growth of tomato seedlings was

significantly different for each of the parameters measured ($p \leq 0.05$) in both trials. Increasing the concentration of the treatments reduced the length of the radicle, plumule and seedling height. Most extracts increased the plumule length at all concentrations in both trials, except for 10 mg/mL where the water extracts of *L. leonurus* and *L. rugosa* significantly reduced the plumule length ($p \leq 0.05$). The development of the plumule is extremely important as this determines if the plant can thrive or survive.

Similar observations with the radicle length and seedling height were identified where effects of *L. leonurus* and *L. rugosa* extracts were the same in both trials with *L. rugosa* being highly inhibitory at 0.2 and 0.4 mg/mL concentrations for radicle length. At 10 mg/mL, *L. leonurus* and *L. rugosa* significantly reduced the height of tomato seedlings while the water extract of *C. anisata* lengthened it. There was no statistical difference in the increase of dry mass for all treatments in both experiments, but the *C. anisata* extract significantly increased the mass of the tomato seedlings in trial 1 at 10 mg/mL. Overall, the investigated plant extracts accelerated seedling growth in length since all the values were less than or comparable to those of the negative control.

Table 6.1: Effect of leaf water extracts of *Clausena anisata*, *Leonotis leonurus* and *Lantana rugosa* on plumule, radicle, height length and dry weight of tomato seedlings (Trial 1)

Treatments	Concentration (mg/mL)	Plumule (cm)	Radicle (cm)	Height (cm)	Dry mass (mg)
Untreated control	Untreated control	0.23±0.01b	0.57±0.03a	0.65±0.01a	0.45±0.01a
C. anisata	0.2	0.30±0.01a	0.52±0.02a	0.65±0.01a	0.33±0.01bc
	0.4	0.24±0.02b	0.42±0.05b	0.50±0.05bc	0.28±0.03c
	10.0	0.25±0.03ab	0.37±0.05bc	0.48±0.06bc	0.37±0.05ab
Untreated control	Untreated control	0.23±0.01b	0.57±0.03a	0.65±0.02a	0.45±0.01a
L. leonurus	0.2	0.25±0.02ab	0.40±0.03b	0.53±0.03bd	0.34±0.02bc
	0.4	0.24±0.02b	0.32±0.03c	0.44±0.04c	0.33±0.03bc
	10.0	0.09±0.02d	0.12±0.03d	0.18±0.05d	0.16±0.04d
Untreated control	Untreated control	0.23±0.01b	0.57±0.03a	0.65±0.02a	0.45±0.01a
L. rugosa	0.2	0.24±0.02b	0.39±0.03b	0.52±0.04bc	0.32±0.03bc
	0.4	0.24±0.02b	0.37±0.03bc	0.52±0.03bc	0.33±0.03bc
	10.0	0.18±0.04c	0.11±0.02d	0.26±0.05d	0.21±0.04d
$Lsd_{p=0.05}$		0.0483	0.0736	0.0877	0.0723
P-value		0.0000	0.0000	0.0001	0.0000
F-value		5.41	5.93	5.04	5.97

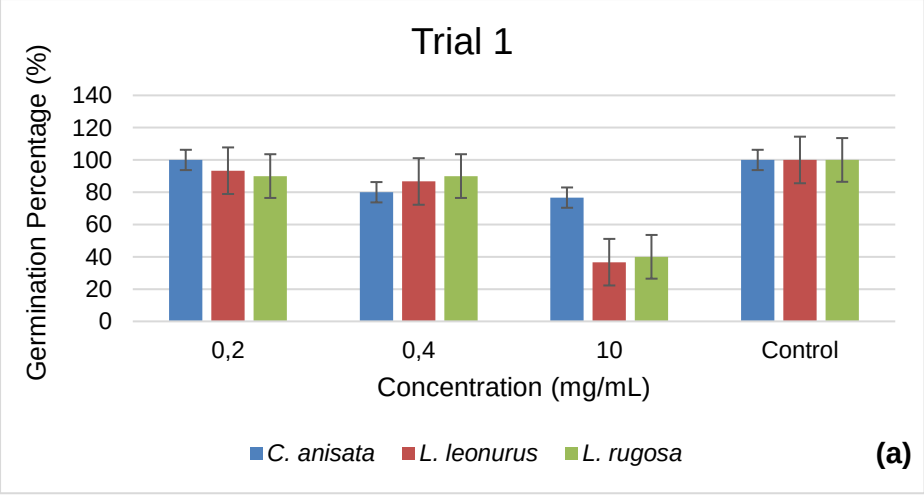
Means are average of 10 replicates, results are presented in mean ± SEM, columns with the same letters are not significantly different P = 0.05.

Table 6.2: Effect of leaf water extracts of *Clausena anisata*, *Leonotis leonurus* and *Lantana rugosa* on plumule, radicle, height length and dry weight of tomato seedlings (Trial 2)

Treatments	Concentration (mg/mL)	Plumule (cm)	Radicle (cm)	Height (cm)	Dry mass (mg)
Untreated control	Untreated control	0.48±0.03ab	0.84±0.04a	0.56±0.03ab	0.30±0.02bcd
C. anisata	0.2	0.41±0.03bc	0.66±0.04bc	0.47±0.03c	0.28±0.02cd
	0.4	0.46±0.03b	0.77±0.04ab	0.49±0.03bc	0.34±0.02abc
	10.0	0.55±0.03a	0.66±0.04bc	0.58±0.03a	0.30±0.02bcd
Untreated control	Untreated control	0.46±0.03b	0.82±0.04a	0.50±0.03bc	0.36±0.02ab
L. leonurus	0.2	0.46±0.03b	0.74±0.04abc	0.50±0.03bc	0.31±0.02bcd
	0.4	0.47±0.03b	0.63±0.04cd	0.52±0.03abc	0.32±0.02abcd
	10.0	0.27±0.03de	0.17±0.04e	0.31±0.03ef	0.19±0.02e
Untreated control	Untreated control	0.33±0.03cd	0.68±0.04bc	0.38±0.03de	0.33±0.02abcd
L. rugosa	0.2	0.42±0.03bc	0.53±0.04d	0.44±0.03cd	0.38±0.02a
	0.4	0.45±0.03b	0.53±0.04d	0.50±0.03abc	0.39±0.02a
	10.0	0.25±0.03e	0.11±0.04e	0.27±0.03f	0.26±0.02d
$Lsd_{P=0.05}$		0.0798	0.1141	0.0843	0.0675
P-value		0.0000	0.0000	0.0000	0.0046
F-value		9.21	10.27	8.16	3.19

Means are average of 10 replicates, results are presented in mean ± SEM, columns with the same letters are not significantly different P = 0.05.

Germination percentage was calculated on the last day of the trial. All extracts at low concentrations (0.2 and 0.4 mg/mL) had stimulatory effects which caused seeds to germinate (plumule increased length in trial 2). Germination in these low concentrations was above 80% in both trials. The higher the final germination percentage the greater the germination of a seed population (Al-Mudaris, 1998). Results shown in trial 2 **(Figure 6.3b)** were better than those in the first trial **(Figure 6.3a)** except for the untreated control. Untreated controls in trial 1 had germination with 100%, while in trial 2 germination was 80% for the *L. rugosa* untreated control. At a high concentration of 10 mg/mL, only *C. anisata* stimulated germination which was statistically higher ($p<0.05$) than the other extracts in both trials. The highest concentration selected in the present study was to motivate for further analysis in the *in vivo* studies as it is difficult to work with low concentrations. The ability of the plants to induce germination of tomato seeds in the present study implies that the plant chemicals had stimulatory effects.



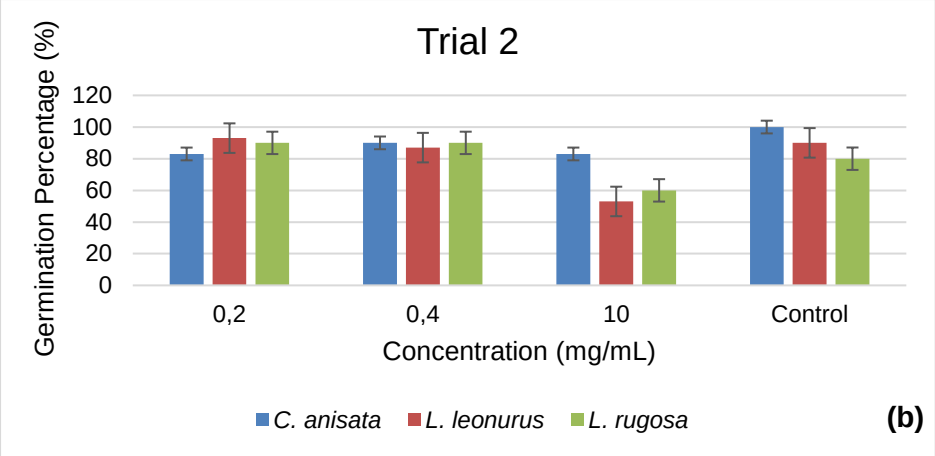


Figure 6.3: The effect of plant extracts on seed germination treated in different concentrations

6.4.2 Glasshouse experiment

The effect of the *L. leonurus* and *C. anisata* powdered material on nematode production and plant growth was investigated and compared to that of *M. angolensis*. There were statistically significant differences (P≤0.05) between the independent trials **(Appendix 6.2)** hence the data was presented separately. **Table 6.3** represents the effect of the treatments on the gall index, J2s and eggs in the roots of tomato plants for trials 1 and 2. The effect of treatments against gall index formation, egg and juvenile production was not statistically different (p≥0.05) from each other in trial 1 and in trial 2 for egg production. There was a significant difference (p≤0.05) in gall formation and juvenile production in trial 2 in all dosages compared to trial 1. *Leonotis leonurus* at 5 g dosage in trial 2 reduced gall formation significantly and its effect was similar to that of Rugby® (positive control).

When compared to the untreated control, the gall index in trial 1 was significantly lower. A ten (10) g dosage was able to reduce the formation of galls in trial 1, while in trial 2

only *L. leonurus* at 5 g was effective and this was not significantly different from Rugby®. Comparison of means for eggs and J2s demonstrated that results from three treatments including *M. angolensis* were not significantly different from each other for both trials including the untreated control, but *C. anisata* at the 15 g dosage effectively reduced the production of eggs as well as J2 in trial 1. Similar observations were made for the 5 g dosage in trial 2, where all treatments reduced the production of eggs, which were not significantly different (P≥0.05) from other dosages, but differences could not be ignored. *Maerua angolensis* actively reduced J2 in the same dosage in 5 g for trial 2. Rugby®, as expected, reduced both the number of eggs as well as J2 in both trials. *Maerua angolensis* as reference plant to the study did not perform better than *L. leonurus* and *C. anisata* in terms of reduction of gall index, and in the production of eggs and J2 of the roots of tomato plant infested with *M. incognita*. Although *M. angolensis* was found to be effective against *M. incognita* in the study of Khosa (2013) and Khosa et al. (2020), in the present study it was not active when compared to *L. leonurus* and *C. anisata.* The good activity shown by *L. leonurus* and *C. anisata* under glasshouse conditions should be further confirmed in microplot experiments and under field conditions where temperature is not controlled. Their potential might also list them under South African plants active against PPN (Makhubu et al., 2021) for possible use in nematode management programmes.

Table 6.3: Effect of powdered material of *L. leonurus*, *C. anisata* and *M. angolensis* (dosage applied as gr of plant material per unit of soil) on gall index, second stage juveniles (J2s) and eggs in roots for trial 1 and trial 2 at 65 days after initiation of treatments

Treatment	Dosage (g)	Trial 1			Trial 2		
		Gall index	Eggs	Juveniles	Gall index	Eggs	Juveniles
Untreated control	5	0.94(7.75)a	4.80(91000)ab	3.52(6000)ab	0.70(4.00)a	4.60(39900)a	2.48(425.00)abc
M. angolensis		0.76(5.25)ab	5.04(176600)a	3.97(9600)a	0.70(4.00)a	3.88(10950)cd	**1.62(125.00)c**
C. anisata		0.78(5.50)ab	5.23(258000)a	3.700(6800)ab	0.64(3.50)a	3.83(9350)d	2.54(625.00)abc
L. leonurus		0.77(5.00)ab	4.80(157200)ab	3.68(10800)ab	**0.53(2.50)b**	3.79(6325)d	3.00(1700.0)a
Rugby ®		0.46(2.00)cd	4.17(27800)abc	3.05(1200)abc	0.48(2.00)b	2.52(350)e	0.00(0.00)d
Untreated control	10	0.65(4.00)bc	4.54(50133)ab	3.58(4533)ab	0.70(4.00)a	4.46(30200)abc	3.14(1575.0)a
M. angolensis		**0.45(2.00)cd**	3.76(16000)abcd	3.34(3400)ab	0.70(4.00)a	4.06(36440)abcd	**1.74(212.50)bc**
C. anisata		**0.48(2.50)cd**	3.67 (7467)abcd	3.42(3200)ab	0.70(4.00)a	3.94(16275)bcd	3.14(2050.0)a
L. leonurus		**0.35(1.25)d**	3.67(13400)abcd	2.55(3600)abc	0.70(4.00)a	4.04(19695)abcd	**1.80(212.50)bc**
Rugby®		0.30(1.00)d	2.25(800)de	2.18(600)bcd	0.48(2.00)b	2.88(850)e	0.00(0.00)d
Untreated control	15	0.55(3.75)bcd	4.00(291400)abc	3.01(8700)abc	0.70(4.00)a	4.03(16963)abcd	2.51(812.50)abc
M. angolensis		0.57(3.00)bcd	3.44(4000)bcd	2.98(1000)abc	0.70(4.00)a	4.29(20575)abcd	2.53(625.00)abc
C. anisata		0.43(1.75)cd	**2.78(6800)cde**	**1.57(800)cd**	0.70(4.00)a	4.04(12125)abcd	2.57(525.00)ab
L. leonurus		0.44(2.25)cd	4.13(33400)abc	2.88(12000)abc	0.70(4.00)a	4.52(36275)ab	3.25(2000.0)a
Rugby®		0.48(2.00)cd	1.63(1200)e	0.73(200)d	0.48(2.00)b	2.53(375)e	0.00(0.00)d
LSDp=0.05		0.2756	1.5888	1.5833	0.0571	0.6243	0.5389
P-value		0.5398	0.6355	0.5225	0.0007	0.1925	0.0317
F-value		0.88	0.76	0.9	4.35	1.48	2.39

Data are means of four replicates; values in brackets are for transformed data; means sharing common letters do not differ significantly (p≥0.05).

The effects of powdered plant material on the plant growth parameters are presented in **Tables 6.4** and **6.5** for trials 1 and 2 respectively. *Meloidogyne incognita* infection did not negatively affect the growth parameters of tomato plants assessed (stem height, wet shoot, dry shoot, number of flowers, stem diameter, fruit number, and fruit mass) except in terms of root mass, fruit number and fruit mass in trial 2. The increase in root mass as a result of gall formation due to nematodes is a negative effect on the host, and also the reduction in root mass by any control method is considered as a negative effect of that treatment on nematodes (Kayani et al., 2012).

In the present study, Rugby® significantly reduced the root mass in all dosages for both trials ($p \leq 0.05$). As for the untreated tomato plants, the mass of the roots was reduced significantly in 15 g dosage ($p \leq 0.05$) in trial 2 as compared to lower dosages and then in 10 and 15 g for trial 1. All the treatments (*M. angolensis*, *C. anisata*, and *L. leonurus*) reduced the weight of roots in the 5 g dosage followed by 15 g in trial 1 while in trial 2, treatments of *C. anisata* and *L. leonurus* at 10 and 15 g dosage significantly reduced the root mass, thus implying phytotoxicity. The effect of Rugby® was also apparent in stem height at 5 g and dry shoot mass as expected. In trial 2, all treatments did not give good results in terms of bearing of fruits and the sizes were much smaller. This could be due to the different times of planting and location that is affected by the temperature. The number of fruits per plant were not more than 5 and the size of fruits was less than 2 g, except for the control at 5 and 15 g, and *C. anisata* at 15 g dosage. Some plants did not bear any fruits, ultimately affecting the mass.

Table 6.4: Effect of *M. angolensis*, *C. anisata* and *L. leonurus* (dosage applied as gr of plant material per unit of soil) on the growth of tomato seedlings infested with *M. incognita* juveniles at 65 days after initiation of treatments in Trial 1.

Treatment	Dosage (g)	Root mass (g)	Stem height (cm)	Wet Shoot (g)	Dry shoot (g)	No. of flowers	Fruit No.	Fruit mass (g)	Stem diameter (cm)
Untreated control	5	103.02abc	2.12(130.50)a	2.36(228.69)a	1.64(42.36)a	22.75abcd	0.82(6.00)bc	159.93a	0.96(8.09)a
M. angolensis		106.60ab	2.08(119.00)a	2.37(239.34)a	1.64(43.00)a	27.25abcd	0.99(9.00)abc	201.83a	1.02(9.58)a
C. anisata		120.12a	2.07(117.25)a	2.36(231.51)a	1.64(43.17)a	29.00ab	0.89(7.25)abc	158.33a	1.02(9.83)a
L. leonurus		90.30abcd	2.10(124.75)a	2.26(182.24)a	1.54(34.08)a	20.50abcd	0.86(6.50)abc	184.52a	0.99(8.78)a
Rugby®		62.65def	2.10(125.00)a	2.31(204.33)a	1.56(35.44)a	28.00abc	0.85(6.50)abc	191.25a	0.96(8.27)a
Untreated control	10	65.70def	2.10(127.25)a	2.31(207.33)a	1.56(36.02)a	18.50abcd	0.86(7.00)abc	166.55a	0.95(8.03)ab
M. angolensis		54.62ef	2.06(114.50)a	2.23(169.55)ab	1.48(29.70)ab	19.00abcd	1.01(9.50)ab	251.76a	0.95(7.86)ab
C. anisata		52.00ef	2.06(115.00)a	2.21(169.29)ab	1.48(30.72)ab	16.50cd	1.02(10.00)ab	212.99a	0.97(8.38)a
L. leonurus		68.18def	2.12 (132.50)a	2.32(210.02)a	1.58(37.29)a	29.50ab	1.02(9.75)ab	190.09a	1.00(9.08)a
Rugby®		45.77f	2.05(113.75)a	2.34(176.04)ab	1.49(30.53)ab	22.75abcd	0.91(7.75)abc	179.17a	0.92(7.25)ab
Untreated control	15	55.72ef	1.54(85.25)b	1.79(181.36)b	1.19(28.93)b	20.25abcd	0.71(6.00)c	160.88a	0.76(7.08)b
M. angolensis		81.13bcde	2.05(112.25)a	2.36(230.52)a	1.60(38.74)a	18.00bcd	0.91(7.25)abc	175.29a	0.95(8.04)ab
C. anisata		84.05bcde	2.10(125.25)a	2.42(263.39)a	1.62(40.88)a	29.50ab	1.05(10.50)ab	229.09a	0.97(8.37)a
L. leonurus		76.77bcdef	2.04(109.00)a	2.31(207.08)a	1.53(33.53)a	16.00d	1.12(13.75)a	258.41a	1.00(9.02)a
Rugby®		69.80cdef	2.05(111.00)a	2.35(230.35)a	1.55(35.94)a	30.00a	0.86(6.50)abc	197.75a	0.95(7.86)ab
LSDp=0.05		33.370	0.3906	0.4615	0.3281	11.712	0.2830	134.11	0.1944
P-value		0.2091	0.3894	0.4008	0.4156	0.1002	0.7167	0.8981	0.7913
F-value		1.44	1.09	1.07	1.05	1.82	0.67	0.43	0.58

Data are means of four replicates; values in brackets are for transformed data; means sharing common letters do not differ significantly ($p \geq 0.05$).

Table 6.5: Effect of *M. angolensis*, *C. anisata* and *L. leonurus* (dosage applied as gr of plant material per unit of soil) on the growth of tomato seedlings infested with *M. incognita* juveniles at 65 days after initiation of treatments in Trial 2.

Data are means of four replicates; values in brackets are for transformed data; means sharing common letters do not differ significantly (p≥0.05).

Treatment	Dosage(g)	Root mass (g)	Stem height (cm)	Wet shoot (g)	Dry shoot (g)	No. of flowers	Fruit No.	Fruit mass (g)	Stem diameter (cm)
Untreated control	5	34.30a	140.05bcd	2.25(178.45)ab	1.41(24.80)ab	18.00bc	0.15(0.75)ab	0.25(2.35)ab	0.19(0.56)bc
M. angolensis		**29.88ab**	137.20cd	2.23(171.65)abc	1.43(25.93)ab	22.50abc	0.00(0.00)b	0.00(0.00)b	0.19(0.57)bc
C. anisata		13.95cde	163.40ab	2.25(181.15)ab	1.38(23.600)abc	15.50c	0.08(0.25)ab	0.10(0.40)b	0.19(0.55)bc
L. leonurus		20.28bcde	147.00abcd	2.25(178.70)ab	1.37(23.38)abc	21.00abc	0.15(0.50)ab	0.24(1.15)b	**0.24(0.74)a**
Rugby®		13.68de	123.50d	2.05(122.58)d	1.16(15.70)d	18.25bc	0.12(0.50)ab	0.10(0.39)b	0.18(0.50)c
Untreated control	10	26.93abc	145.53abcd	2.27(187.50)ab	1.41(25.30)ab	25.75abc	0.00(0.00)b	0.00(0.00)b	0.10(0.57)bc
M. angolensis		27.73ab	155.85abc	2.30(201.52)a	1.41(25.13)ab	25.00abc	0.00(0.00)b	0.00(0.00)b	0.20(0.60)abc
C. anisata		**25.33abcd**	154.65abc	2.32(211.58)a	1.47(28.88)a	24.75abc	0.08(0.25)ab	0.10(0.39)b	0.22(0.65)ab
L. leonurus		**21.13abcde**	161.90abc	2.31(205.58)a	1.46(27.90)a	26.75ab	0.12(0.50)ab	0.19(1.15)b	0.19(0.57)bc
Rugby®		13.58de	159.65abc	2.08(129.88)cd	1.14(14.55)d	17.75bc	0.08(0.25)ab	0.09(0.30)b	0.18(0.52)bc
Untreated control	15	12.60de	150.90abc	2.17(153.13)abcd	1.25(18.08)bcd	15.75c	0.23(1.00)ab	0.35(3.15)ab	0.20(0.59)abc
M. angolensis		**28.63ab**	150.15abc	2.31(201.97)a	1.46(28.03)a	29.25a	0.00(0.00)b	0.00(0.00)b	0.20(0.59)abc
C. anisata		20.68bcde	160.95abc	2.26(182.70)ab	1.41(24.88)ab	23.25abc	0.12(0.50)ab	0.20(1.30)b	0.19(0.55)bc
L. leonurus		16.95bcde	157.55abc	2.31(204.17)a	1.42(25.55)ab	22.75abc	0.31(1.25)a	0.64(5.45)a	0.21(0.64)abc
Rugby®		10.33e	169.50a	2.15(147.08)bcd	1.20(15.83)cd	19.50abc	0.00(0.00)b	0.00(0.00)b	0.18(0.51)bc
LSd p= 0.05		13.180	25.024	0.1546	0.2014	10.534	0.2392	0.3947	0.0390
P-value		0.2001	0.2218	0.8368	0.7599	0.6282	0.6561	0.5558	0.4615
F-value		1.46	1.41	0.52	0.62	0.77	0.74	0.86	0.98

6.5 Discussion

The results from the seedling germination study concerning the growth of tomato seedlings after incubation with the investigated plant extracts indicated that lower concentrations stimulated the seedling growth in terms of plumule in trial 2 and increased the dry weight, but higher concentrations had suppressive effects. Though the mass of the dried shoot was high, the phytotoxic effects resulting from the increased concentration could not be ignored. The negative effects of high concentrations on the development of plants was also reported by Okeke et al. (2015), where the water leaf extract of *Morinda lucida* reduced the height and dry weight of *Amaranthus spinosus* and *Amaranthus hybridus*. In the present study, plumule length was significantly reduced at the lower concentrations of *L. rugosa.* The negative effects with lower concentrations were also reported by Fikreyesus et al. (2011) on tomato when treated with water leaf extracts of *Eucalyptus camaldulensis*. As reported by Adepoju et al. (2018), stimulation of growth by plant extracts could be due to the breaking down of functional allelochemicals in the extracts and their subsequent transformation to plant nutrients required for growth. The allelochemicals can also have inhibitory effects that prevent the growth of the plant. Generally, the presence of phytochemicals in the water extracts of *L. leonurus*, *C. anisata* and *L. rugosa* might have contributed to the stimulatory effects observed at low concentrations ($p<0.05$) which assisted the growth of tomato seedlings, while higher concentrations ($p>0.05$) had inhibitory effects.

To successful manage PPN and protect crops from phytotoxicity, allelochemicals should be applied in safe concentrations (Mashela et al., 2015). Root gall index is an important parameter used when evaluating the effect of treatments and the level of

infestation of *M. incognita* (Bridge and Page, 1980). Plant growth increase and nematode reduction were not necessarily correlated to the dosages of the evaluated plants, but this was not applicable to all the measured parameters. The strong significance in gall index for trial 2 does not necessarily mean that the treatments were effective since they were comparable to the untreated control, and this was different for trial 1. Though the plants were active *in vitro*, their activity under glasshouse conditions was average. This agrees with the findings of Wen et al. (2017) where the extract of *Gleditsia sinensis* did not suppress galling or *M. incognita* egg population densities on pepper in the greenhouse, and their powdered fruit meal as a soil amendment did not reduce the numbers of galls/g root and/or eggs/g root on pepper and water spinach as compared to the good results of the *in vitro* tests.

As reported by Galbieri and Belot (2016) cited in Ebone et al. (2019), the developmental stage of nematode, environmental temperature and the nature of chemicals present affect the performance of nematicides. *Clausena anisata* was effective in reducing the number of J2 and eggs in the roots of tomato plants at a high dosage, while *L. leonurus* was only active in reducing juveniles at 10 g. Maximum reduction against *M. incognita* was reported by Kayani et al. (2012), where *Cannabis sativa* reduced egg mass by 62.36% and *Zanthoxylum alatum* with 56.84% at highest dosage of 20 g. The high concentration might not be realistic for use by farmers and can be phytotoxic to the crops. The proposed low quantity of 5 g material is required for use in smallholder farming to mitigate the drawbacks of conventional organic amendments via GLT method (Mashela, 2002; Mashela and Nthangeni, 2002). Drawbacks include supply of material and their quality, as well as inability to deliver large quantities to the field (McSorley and Gallaher, 1995). A small number of J2 were

counted in trial 2 compared to trial 1, and this could mean that even if some of them penetrated the plant, they may not cause economic damage to the plant due to their insignificant number (Kayani et al., 2012). Reduction of egg numbers was also observed at all dosages, and though the level of significance was small, it could not go unnoticed in both trials. The reduction caused by the evaluated plants in trial 2 at lower dosages could lead to a decrease in secondary inoculum of the nematode. Khan et al. (2019) stated that poor penetration of J2, retarded feeding and the reproductive activity of RKN also play vital role in nematode reduction. Several studies have reported good activity of plants under glasshouse conditions (Tsay et al., 2004; Kumar and Khanna, 2006; Natarajan et al., 2006; Thovhakale et al., 2006; Khosa, 2013; Bawa et al., 2014; Malungane, 2016).

According to Cheng (1992) cited by Kokalis-Burelle and Rodríguez-Kàbana (2006), allelochemicals are easily metabolized and/ or hydrolyzed, and for them to be effective they need plants to be actively growing and be secreted into the rhizosphere. Plant growth and yield did not differ between the untreated control and the applied treatments, thus demonstrating a lack of phytotoxicity and a lack of fertilizer effect in tomato at the applied rates. The insignificant difference between treatments and the untreated control was mainly observed with regard to the plant height, wet shoot mass, dry shoot mass, number of flowers, fruit mass and stem diameter of the tomato plants. The lack of phytotoxicity and fertilizer effect on tomato was also observed by Mashela (2002) when using *Cucumis myriocarpus* fruits as soil amendment for *M. incognita* in microplot conditions. Variation in reduction of *M. incognita* J2 and eggs, growth parameters, and the mass of tomato plants in two sets of repeated trials may be due to the different times of planting and location which may affect the temperature, direct

sunlight, and soil moisture content for plant growth and nematode reproduction in the greenhouse.

Conclusion

Leonotis leonurus, *Clausena anisata* and *Lantana rugosa* water extracts were not phytotoxic to the growth of tomato seedlings at all tested concentrations under *in vitro* conditions. Lower concentrations of plant extracts stimulated germination, whereas high concentrations had inhibitory effects against the growth of tomato seedlings except in the case of *C. anisata.* The non-phytotoxic effects demonstrated by the investigated plants at the tested concentrations *in vitro* may endorse further analysis on the plants *in vivo*. However, high concentrations should be further investigated for *L. leonurus* and *L. rugosa* as these might delay seedling development.

In the treatment of tomato plants infested with *M. incognita,* the plants had promising ability to reduce gall index, number of eggs and J2 when compared to the untreated control and the reference plant material tested (*M. angolensis*) which had similar activity in most cases. There was a lack of phytotoxic and fertilizer effect on tomato plants at applied rates on the growth parameters, since there was little difference to the untreated control. The phytochemicals in the plants might be active, however with low effects under greenhouse conditions. The promising *in vitro* activity of *L. leonurus* and *C. anisata* can therefore most likely not be exploited in the form of powder in the field. It may be suggested that the plants of interest should be extracted with water, as good anthelmintic activity *in vitro* was obtained with water extracts (**Chapter 4**, **Table 4.1)**. These extracts should be evaluated under glasshouse, microplot and field conditions to confirm their activity against PPN infestations.

CHAPTER 7

General Discussion, Conclusion and Recommendations

The aim of this study was to determine the anthelmintic efficacy of selected non-crop plant species in an effort to identify active natural protective agents against root-knot nematodes (RKN) and phytopathogenic microbes infecting tomatoes. The aim incorporated an investigation of the correlation of activity of plant extracts against free-living nematodes (FLN) and animal-parasitic nematodes (APN) with activity against plant-parasitic nematodes (PPN). To achieve the aim of this project, several objectives were formulated and results obtained are discussed below.

7.1 Discussion

7.1.1 Comparative anthelmintic activity against FLN and APN: Objective 1

The first objective was to test plants previously reported to have anthelmintic activity against free-living *Caenorhabditis elegans* and the ruminant parasite *Haemonchus contortus,* and to determine their chemical constituents. Generally, all plant extracts prepared using water, acetone and DCM/MeOH investigated in the present study had good anthelmintic activity against *C. elegans* and *H. contortus*. When fractions of different polarity of selected plant extracts were tested for motility against *C. elegans*, good activity was observed at the lowest concentration tested, and the activity was higher than that of the original crude extracts, thus fractionation potentiated the activity. Different observations were made with activity of fractions against *H.*

contortus, as they were less effective when compared to the extracts. The inactivity of the fractions in the *H. contortus* Infective larvae hatch assay indicated that fractionation could not potentiate the activity of fractions.

Testing plants for cytotoxicity *in vitro* allows identification and prioritisation of plant extracts useful for further biological evaluation. The *in vitro* safety of the plant extracts indicated that all the fractions and plant extracts of *Leonotis leonurus* and *Clausena anisata* were safe against Vero African green monkey kidney cells at the concentrations tested, while *Lantana rugosa* extracts were toxic with selectivity index (SI) value lower than 1.

The profiled chemicals based on GC-MS analysis revealed the presence of various chemicals which could contribute to the activity shown by different extracts against *C. elegans* and *H. contortus*. There was a good relationship between anthelmintic activity against APN and FLN, supporting the use of *C. elegans* as a good test organism for anthelmintic activity investigations. The weak correlation between motility activity against *C. elegans* and *M. incognita* suggested that *C. elegans* is not a good model organism for PPN in the present study. However, similar concentrations should be used for testing correlations, and other experiments such as larval development inhibition should be done to confirm the activity. Moderate correlations were observed in hatch inhibition activity against APN and PPN, implying that plants with activity against animal nematodes can also be active against PPN.

7.1.2 Investigating the potential of South African plants against soilborne pathogens: Objective 2

The second objective of the study was to screen all the plant extracts against phytopathogens infecting tomato as well as root-knot nematodes (RKN). Soilborne pathogens are economically important, causing great losses in agricultural production globally. *Meloidogyne* infection affects translocation of water and nutrients in the root system and some phytopathogens such as bacteria and fungi may enter the xylem and disrupt the movement of water, thus causing extensive damage to the crop (Jahr et al., 1999). In this study, the strongest and broadest spectrum of antimicrobial activity was observed with the acetone extracts, followed by DCM/MeOH extracts. Extracts prepared using water showed weak activity with the exception of the *L. leonurus* extract against *Fusarium oxysporum*. Acetone extracts of *S. lancea* and *L. sericea* were effective at low concentrations in most bacteria tested. Extracts of *C. orbiculata*, *S. lancea* and *L. leonurus s*howed antifungal activity. The most susceptible organisms in the present study were *R. pseudosolanacearum, R. solanacearum* and *X. vesicatoria. Ralstonia* and *Xanthomonas* species are associated with roots of tomatoes and since nematodes cause injury to the roots of the host plants, the ability of the extracts to inhibit the growth of the studied phytopathogens may assist in managing bacterial and fungal pathogens causing damage to crops.

Leonotis leonurus had good activity in the J2 hatch inhibition and motility assays with *Meloidogyne incognita* while *C. anisata* was only active in inhibiting J2 from hatching. The good activity of *L. leonurus* and *C. anisata in vitro* against juveniles of RKN as assessed by effects on their motility encourages further investigation regarding the

isolation of nematicidal compounds as well as *in vivo* glasshouse trials to confirm the activity.

7.1.3 Isolation and characterisation of nematicidal compounds from *Leonotis leonurus:* Objective 3

Owing to the good nematicidal activity shown by *L. leonurus* against *M. incognita* as well as activity against *H. contortus*, the third objective was to isolate and characterise active compounds from the plant. Bioassay-guided fractionation was used to isolate nematicidal compounds from the acetone leaf extract of *L. leonurus* using *C*. *elegans* as test organism. The dichloromethane fraction was selected for further purification since it showed good activity against motility and J2 hatching of *M. incognita*. Fractions from column chromatography were tested, and this led to isolation of seven compounds. Due to low quantities isolated, only leoleorin C, a known compound previously isolated from the leaves of *L. leonurus* by Naidoo et al. (2011), was characterised and identified. In the present study, the compound showed promising activity against *Clavibacter michiganensis subsp. michiganensis* when evaluated using bioautography. This is the first study reporting the activity of the compound against phytopathogens infecting tomato, as well as activity against *M. incognita.*

7.1.4 *In vitro* testing on the growth of tomato seedlings and activity against *M. incognita* under glasshouse conditions: Objective 4

Plant extracts of *L. leonurus*, *L. rugosa* and *C. anisata* were tested for their phytotoxic effects on the growth of tomato seedlings. This was done following their good activity against J2 motility and J2 hatching of *M. incognita* and, since they have potential for the development of nematicidal agents for crop protection, it is important to test their effects on seedlings. The water extracts from the tested plants indicated that lower concentrations stimulated the seedling growth by increasing the height of the plumule, radicle, overall height of the tomato seedling and also increased the dry weight, but higher concentrations had suppressive effects on these parameters. Lower concentrations of all plant extracts stimulated germination, whereas high concentrations had inhibitory effects, except in the case of *C. anisata.*

Powdered material of *C. anisata* and *L. leonurus* dried leaves were further evaluated against reproduction of *M. incognita* under glasshouse conditions. This followed the good nematicidal activity (J2 motility and J2 hatch inhibition) shown *in vitro*. The aim was to see if promising *in vitro* results could translate to *in vivo* efficacy. The plants had the ability to reduce the gall index, as well as numbers of eggs and second-stage juveniles (J2) when compared to the untreated control. There was a lack of phytotoxic effect but also a lack of fertilizer effect on tomato plants at applied rates since the growth parameters of treated plants were comparable to those of the untreated controls. Thus, *M. incognita* infection did not negatively affect the growth parameters assessed (stem height, wet shoot, dry shoot, number of flowers, stem diameter, fruit number, and fruit weight). This could mean that there was poor penetration of J2 and

this ultimately led to few nematodes multiplying, hence the tomato plants did not show any symptoms. One other reason for this might be the differing average temperatures in the different provinces which may also affect the reproduction of RKN. It is important to conduct such studies in different locations to determine the efficacy of the treatments and further analysis can be done in microplots and in field conditions to confirm the activity. In the current study, various phytochemicals in the plants might be active, however with low effect. For this reason, powdered material might not be a good solution for controlling RKN, and extracts of the plants should be investigated for anthelmintic effects *in vivo* to assess their full potential.

7.2 Conclusion

Plants used in the present study which have been previously reported to have anthelmintic activity against free-living and animal-parasitic nematodes have nematicidal potential against the plant-parasitic nematode (PPN) species *M. incognita*. In confirming the activity against free-living *C. elegans* and animal-parasitic *H. contortus*, use of different extraction solvents did not affect the overall results since all plants had activity. When testing for correlations in activity, efficacy against *C. elegans* was strongly correlated with activity against animal-parasitic nematodes, but there was little correlation with activity against PPN.

It was also shown that plant species with nematicidal activity investigated in this study were generally not active against the selected phytopathogenic bacteria and fungi tested, or had relatively weak activity. Fractionation of the extracts based on polarity

increased the activity against *C. elegans*. This could mean that since activity against FLN correlated reasonably with activity against APN in this study, the fractions active against *C. elegans* can be exploited in developing anthelmintic agents. The compound isolated and identified from *L. leonurus*, leoleorin C, was not active against *C. elegans,* a model organism used for bioassay-guided isolation. However, fractions from where this compound and others were obtained were active against the FLN. A combination of compounds from this plant are most likely working in synergy. There is great potential to develop products from *L. leonurus* and *C. anisata* plants because of their promising efficacy both *in vitro* and *in vivo*, and their low toxicity against Vero cell lines and lack of phytotoxic effects on tomato seedlings.

7.3 Recommendations for future research

For development of nematicidal products, there are several processes that need to be followed before a product can be made available. In the future, the following areas of research may be investigated:

- There appears to be a low likelihood of developing a single compound with nematicidal activity from *L. leonurus*. Compounds as well as the extracts might be working in synergy or they might be using different, complementary mechanisms. It would be extremely useful to evaluate the synergism of various constituent compounds of the studied plants. This might enhance activity and the mixture can be used to develop a product with broad-spectrum anthelmintic and phytopathogenic activity.
- It would be advisable to use *M. incognita* as the test species for bioassay-guided isolation of nematicidal compounds. Although it is costly it may be a

worthwhile exercise to obtain desired results with compounds responsible for activity since the free-living model organism did not provide a good correlation with activity against the PPN.

- It is also important to conduct mechanism of action studies on the active crude extracts as well as the isolated compounds to determine whether these differ from currently used nematicides.
- Fractionation of the plant extracts enhanced activity under *in vitro* conditions. It would be useful if the fractions, or combinations of the most active fractions, could also be tested *in vivo* under glasshouse conditions. This would help to formulate products that are highly effective and that perhaps function effectively based on a variety of complementary mechanisms against nematodes and phytopathogenic microbes.
- Powdered material from the plants was not highly effective in the *in vivo* nematicidal trials in this study, so it would be interesting to test water extracts since these were active *in vitro* to investigate the correlation between *in vivo* and *in vitro* efficacy.
- Microplots and field trials are highly recommended as a further step in development of active plant-based preparations able to protect crops against phytopathogenic nematodes and microorganisms.

References

Abad, P., Gouzy, J., Aury, J.-M., Castagnone-Sereno, P., Danchin, E.G., Deleury, E., Perfus-Barbeoch, L., Anthouard, V., Artiguenave, F., Blok, V.C., 2008. Genome sequence of the metazoan plant-parasitic nematode *Meloidogyne incognita*. Nature Biotechnology 26, 909-915.

Abbott, W.S., 1925. A method of computing the effectiveness of an insecticide. Journal of Economic Entomology 18, 265-267.

Abdel-Rahman, F.H., Alaniz, N.M., Saleh, M.A., 2013. Nematicidal activity of terpenoids. Journal of Environmental Science and Health, Part B 48, 16-22.

Abdel-Rahman, F.H., Clark, S., Saleh, M.A., 2008. Natural organic compounds as alternative to methyl bromide for nematodes control. Journal of Environmental Science and Health, Part B 43, 680-685.

Abiola, O.A., 2020. Vegetable production and nematodes infestation: Impacts on small-scale farming communities of South Africa. World Journal of Advanced Research and Reviews 7, 168-177.

Adamu, M., 2013.The efficacy of traditionally used *Leucosidea sericea* (Rosaceae) against *Haemonchus contortus* and microbial pathogens. PhD Dissertation, University of Pretoria.

Adamu, M., Naidoo, V., Eloff, J.N., 2013. Efficacy and toxicity of thirteen plant leaf acetone extracts used in ethnoveterinary medicine in South Africa on egg hatching and larval development of *Haemonchus contortus*. BMC Veterinary Research 9, 38.

Adamu, M., Naidoo, V., Eloff, J.N., 2014. The antibacterial activity, antioxidant activity and selectivity index of leaf extracts of thirteen South African tree species used in ethnoveterinary medicine to treat helminth infections. BMC Veterinary Research 10, 1-7.

Adedapo, A.A., Sofidiya, M.O., Maphosa, V., Moyo, B., Masika, P.J., Afolayan, A.J., 2008. Anti-inflammatory and analgesic activities of the aqueous extract of *Cussonia paniculata* stem Bark. Records of Natural Products 2, 46.

Adepoju, I., Oluwatayo, J., Iheukwumere, C., Ekefan, E., 2018. Allelopathic effect of *Hibiscus sabdariffa* on the yield of some local best cultivars of white yam in nematode infested soil. Fuw Trends In Science & Technology Journal 3, 1029-1031.

Afzal, I., Munir, F., Ayub, C., Basra, S., Hameed, A., Nawaz, A., 2009. Changes in antioxidant enzymes, germination capacity and vigour of tomato seeds in response of priming with polyamines. Seed Science and Technology 37, 765-770.

Aganga, A., Mosase, K., 2001. Tannin content, nutritive value and dry matter digestibility of *Lonchocarpus capassa*, *Zizyphus mucronata*, *Sclerocarya birrea*, *Kirkia acuminata* and *Rhus lancea* seeds. Animal Feed Science and Technology 91, 107-113.

Agbenin, O.N., Marley, P., 2006. *In vitro* assay of some plant extracts against *Fusarium oxysporum* f. sp. *lycopersici* causal agent of tomato wilt. Journal of Plant Protection Research 46, 215-220.

Agnihotri, V.K., ElSohly, H.N., Smillie, T.J., Khan, I.A., Walker, L.A., 2009. Constituents of *Leonotis leonurus* flowering tops. Phytochemistry Letters 2, 103-105.

Agrios, G.N., 2005. Plant pathology. Elsevier academic press Amsterdam.
Agyepong, N., Agyare, C., Adarkwa-Yiadom, M., Gbedema, S.Y., 2014. Phytochemical investigation and anti-microbial activity of *Clausena anisata* (Willd), Hook. African Journal of Traditional, Complementary and Alternative Medicines 11, 200-209.
Agyepong, N., Agyare, C., Ossei, P.P.S., Boakye, Y.D., 2015. Antioxidant and *in vivo* wound healing activities of *Clausena anisata*. European Journal of Medicinal Plants 10, 1-8.
Ahmed, M., Laing, M., Nsahlai, I., 2013. *In vitro* anthelmintic activity of crude extracts of selected medicinal plants against *Haemonchus contortus* from sheep. Journal of Helminthology 87, 174.
Akhtar, M., Malik, A., 2000. Roles of organic soil amendments and soil organisms in the biological control of plant-parasitic nematodes: a review. Bioresource Technology 74, 35-47.
Aktar, M.W., Sengupta, D., Chowdhury, A., 2009. Impact of pesticides use in agriculture: their benefits and hazards. Interdisciplinary Toxicology 2, 1-12.
Al-Mudaris, M., 1998. Notes on various parameters recording the speed of seed germination. Der Tropenlandwirt-Journal of Agriculture in the Tropics and Subtropics 99, 147-154.
Ali, M.A., Abbas, A., Kreil, D.P., Bohlmann, H., 2013. Overexpression of the transcription factor RAP2. 6 leads to enhanced callose deposition in syncytia and enhanced resistance against the beet cyst nematode *Heterodera schachtii* in Arabidopsis roots. BMC Plant Biology 13, 1-17.
Amabeoku, G., Green, I., Kabatende, J., 2007. Anticonvulsant activity of *Cotyledon orbiculata* L.(Crassulaceae) leaf extract in mice. Journal of Ethnopharmacology 112, 101-107.
Amabeoku, G.J., Kabatende, J., 2011. Antinociceptive and anti-inflammatory activities of leaf methanol extract of *Cotyledon orbiculata* L.(Crassulaceae). Advances in Pharmacological Sciences 2012, 1-6.
Amoo, S.O., Aremu, A.O., Moyo, M., Van Staden, J., 2012. Antioxidant and acetylcholinesterase-inhibitory properties of long-term stored medicinal plants. BMC Complementary and Alternative Medicine 12, 1-9.
Amusan, O.O., 2008. Ethical and environmental issues in bioprospecting for drugs through traditional medicine: the case of Swaziland. The African Journal of Plant Sciences and Biotechnology 2, 1-9.
Andrés, M.F., González-Coloma, A., Sanz, J., Burillo, J., Sainz, P., 2012. Nematicidal activity of essential oils: a review. Phytochemistry Reviews 11, 371-390.
Anju, A., Ravi S, P., Bechan, S., 2010. Water pollution with special reference to pesticide contamination in India. Journal of Water Resource and Protection 2010.
ARC. 2013. *Agricultural Research Council. 2013. ARC Annual Report 2013/2014* [Online]. Available: https://www.arc.agric.za [Accessed 18 September 2019].
Aremu, A., Ndhlala, A., Fawole, O., Light, M., Finnie, J., Van Staden, J., 2010. *In vitro* pharmacological evaluation and phenolic content of ten South African medicinal plants used as anthelmintics. South African Journal of Botany 76, 558-566.
Aremu, A.O., Amoo, S.O., Ndhlala, A.R., Finnie, J.F., Van Staden, J., 2011. Antioxidant activity, acetylcholinesterase inhibition, iridoid content and mutagenic evaluation of *Leucosidea sericea*. Food and Chemical Toxicology 49, 1122-1128.

Aremu, A.O., Finnie, J.F., Van Staden, J., 2012. Potential of South African medicinal plants used as anthelmintics – Their efficacy, safety concerns and reappraisal of current screening methods. South African Journal of Botany 82, 134-150.

Askary, T.H., 2008.Studies on root-knot nematodes infesting pigeonpea and its integrated management. PhD Dissertation, Aligarh Muslim University.

Ayisi, N.K., Nyadedzor, C., 2003. Comparative *in vitro* effects of AZT and extracts of *Ocimum gratissimum*, *Ficus polita, Clausena anisata*, *Alchornea cordifolia,* and *Elaeophorbia drupifera* against HIV-1 and HIV-2 infections. Antiviral Research 58, 25-33.

Bailey, R. 2020. *Nematoda: Roundworms* [Online]. Available: https://www.thoughtco.com/nematoda-free-living-parasitic-roundworms-4123864 [Accessed 14 February 2020].

Bawa, J.A., Mohammed, I., Liadi, S., 2014. Nematicidal effect of some plants extracts on root-knot nematodes (*Meloidogyne incognita*) of Tomato (*Lycopersicon esculentum*). World Journal of Life Sciences and Medical Research 3, 81.

Beourou, S., Le Lamer, A.-C., Maurel-Chevalley, S., Mutiso, P.B.C., Souard, F., Moulis, C., 2013. Evaluation of the antiplasmodial activity of extracts of plants used in traditional medicine in Kenya. International Journal of Medicinal Plants Research 2, 219-224.

Besier, R., Kahn, L., Sargison, N., Van Wyk, J., 2016. Diagnosis, treatment and management of *Haemonchus contortus* in small ruminants. Advances in Parasitology 93, 181-238.

Bhat, R.B., Jacobs, T.V., 1995. Traditional herbal medicine in Transkei. Journal of Ethnopharmacology 48, 7-12.

Bhatt, B., Todaria, N., 1990. Studies on the allelopathic effects of some agroforestry tree crops of Garhwal Himalaya. Agroforestry Systems 12, 251-255.

Bienvenu, E., Amabeoku, G., Eagles, P., Scott, G., Springfield, E., 2002. Anticonvulsant activity of aqueous extract of *Leonotis leonurus*. Phytomedicine 9, 217-223.

Bird, D.M., Kaloshian, I., 2003. Are roots special? Nematodes have their say. Physiological and Molecular Plant Pathology 62, 115-123.

Bisi-Johnson, M., Obi, C., Kambizi, L., Nkomo, M., 2010. A survey of indigenous herbal diarrhoeal remedies of OR Tambo district, Eastern Cape Province, South Africa. African Journal of Biotechnology 9.

Blaxter, M., Koutsovoulos, G., 2015. The evolution of parasitism in Nematoda. Parasitology 142, S26–S39.

Blaxter, M.L., De Ley, P., Garey, J.R., Liu, L.X., Scheldeman, P., Vierstraete, A., Vanfleteren, J.R., Mackey, L.Y., Dorris, M., Frisse, L.M., 1998. A molecular evolutionary framework for the phylum Nematoda. Nature 392, 71-75.

Bleve-Zacheo, T., Melillo, M.T., Castagnone-Sereno, P., 2007. The contribution of biotechnology to root-knot nematode control in tomato plants. Pest Technology 1, 1-16.

Bogner, C.W., Kamdem, R.S., Sichtermann, G., Matthäus, C., Hölscher, D., Popp, J., Proksch, P., Grundler, F.M., Schouten, A., 2017. Bioactive secondary metabolites with multiple activities from a fungal endophyte. Microbial Biotechnology 10, 175-188.

Bonnie, P. 2021. The importance of healthy roots. Bonnie Plants.

Bosman, A., Combrinck, S., Roux-Van der Merwe, R., Botha, B., McCrindle, R., Houghton, P., 2004. Isolation of an anthelmintic compound from *Leucosidea sericea*. South African Journal of Botany 70, 509-511.

Brenner, S., 1974. The genetics of *Caenorhabditis elegans*. Genetics 77, 71-94.

Bridge, J., Page, S., 1980. Estimation of root-knot nematode infestation levels on roots using a rating chart. International Journal of Pest Management 26, 296-298.

Burke, J., Kaplan, R., Miller, J., Terrill, T., Getz, W., Mobini, S., Valencia, E., Williams, M., Williamson, L., Vatta, A., 2007. Accuracy of the FAMACHA system for on-farm use by sheep and goat producers in the southeastern United States. Veterinary Parasitology 147, 89-95.

Burkhart, C.N., 2000. Ivermectin: an assessment of its pharmacology, microbiology and safety. Veterinary and Human Toxicology 42, 30-35.

Campbell, W., Fisher, M., Stapley, E., Albers-Schonberg, G., Jacob, T., 1983. Ivermectin: a potent new antiparasitic agent. Science 221, 823-828.

Caromel, B., Gebhardt, C., 2011. Breeding for nematode resistance: Use of genomic information. In: Jones J., Gheysen G. & C., F (eds.) Genomics and molecular genetics of plant-nematode interactions. Dordrecht: Springer.

Chakraborty, A., Chowdhury, B., Bhattacharyya, P., 1995. Clausenol and clausenine—two carbazole alkaloids from *Clausena anisata*. Phytochemistry 40, 295-298.

Chitwood, D.J., 2002. Phytochemical based strategies for nematode control. Annual Review of Phytopathology 40, 221-249.

Clarkson, C., Maharaj, V.J., Crouch, N.R., Grace, O.M., Pillay, P., Matsabisa, M.G., Bhagwandin, N., Smith, P.J., Folb, P.I., 2004. *In vitro* antiplasmodial activity of medicinal plants native to or naturalised in South Africa. Journal of Ethnopharmacology 92, 177-191.

Cohen, J., 1988. Statistical power analysis for the behavioral sciences. L. Erlbaum Associates, Hillsdale, N.J.

Colegate, S.M., Molyneux, R.J., 2007. Bioactive natural products: detection, isolation, and structural determination. CRC press,

Coles, G., Bauer, C., Borgsteede, F., Geerts, S., Klei, T., Taylor, M., Waller, P., 1992. World Association for the Advancement of Veterinary Parasitology (WAAVP) methods for the detection of anthelmintic resistance in nematodes of veterinary importance. Veterinary Parasitology 44, 35-44.

Collange, B., Navarrete, M., Peyre, G., Mateille, T., Tchamitchian, M., 2011. Root-knot nematode (*Meloidogyne*) management in vegetable crop production: The challenge of an agronomic system analysis. Crop Protection 30, 1251-1262.

Conrad's, J. 2012. Nematodes: a tomato disaster. *Naturalist Newsletter*.

Cooper, K.M., McMahon, C., Fairweather, I., Elliott, C.T., 2015. Potential impacts of climate change on veterinary medicinal residues in livestock produce: An island of Ireland perspective. Trends in Food Science & Technology 44, 21-35.

Cordell, G.A., Farnsworth, N.R., Beecher, C.W., Soejarto, D.D., Kinghorn, A.D., Pezzuto, J.M., Wall, M.E., Wani, M.C., Brown, D.M., O'Neill, M.J., 1993. Novel strategies for the discovery of plant-derived anticancer agents. In: Kinghorn, DA & Balandrin, MF (eds.) Human Medicinal Agents from Plants. American Chemical Society.

Costa, C., Bevilaqua, C., Camurça-Vasconcelos, A., Maciel, M., Morais, S., Castro, C., Braga, R., Oliveira, L., 2008. *In vitro* ovicidal and larvicidal activity of

Azadirachta indica extracts on *Haemonchus contortus*. Small Ruminant Research 74, 284-287.
Costa, J.C., Lilley, C.J., Atkinson, H.J., Urwin, P.E., 2009. Functional characterisation of a cyst nematode acetylcholinesterase gene using *Caenorhabditis elegans* as a heterologous system. International Journal for Parasitology 39, 849-858.
Cunningham, A., 1988. An investigation of the herbal medicine trade in Natal/KwaZulu. University of Natal, Institute of Natural Resources, University of Natal, Pietermaritzburg.
D'Addabbo, T., Argentieri, M.P., Radicci, V., Grassi, F., Avato, P., 2017. *Artemisia annua* compounds have potential to manage root-knot and potato cyst nematodes. Industrial Crops and Products 108, 195-200.
da Silva, C.M.A., da Silva Costa, B.M., da Silva, A.G., de Souza, E.B., da Silva, M., Vanusa, a., dos Santos Correia, M.T., de Menezes, L.V.L., ucia. 2016. Antimicrobial activity of several Brazilian medicinal plants against phytopathogenic bacteria. African Journal of Microbiology Research 10, 578-583.
DAFF. 2015. *Department of Agriculture, Forestry and Fisheries. Annual Report 2015-2016* [Online]. Available: http://www.daff.gov.za/ [Accessed 15 August 2019].
DAFF 2019. A profile of the South African tomato market value chain.
Danahap, L., Wonang, D., 2016. Antinematicidal efficacy of root exudates of some *Crotalaria* species on *Meloidogyne incognita* (root-knot nematode)(Kofoid and White) Chitwood isolated from infected *Lycopersicum esculentum* L.(tomato) plant. International Journal of Science Technology Research 5, 79-84.
Dari, L., Addo, A., Dzisi, K.A., 2016. Pesticide use in the production of Tomato (*Solanum lycopersicum* L.) in some areas of Northern Ghana. African Journal of Agricultural Research 11, 352-355.
Dellavalle, P.D., Cabrera, A., Alem, D., Larrañaga, P., Ferreira, F., Rizza, M.D., 2011. Antifungal activity of medicinal plant extracts against phytopathogenic *fungus Alternaria* spp. Chilean Journal of Agricultural Research 71, 231-239.
Dold, A., Cocks, M., 2001. Traditional veterinary medicine in the Alice district of the Eastern Cape Province, South Africa: Research in action. South African Journal of Science 97, 375-379.
Doughari, J.H., Ndakidemi, P.A., Human, I.S., Benad, S., 2012. Multi-drug resistance, verotoxin production and efficacy of crude stem bark extracts of *Curtisia dentata* among *Escherichia coli* (non-O157) and *Acinetobacter* species isolates obtained from water and wastewater samples African Journal of Pharmacy and Pharmacology 6, 1653-1667.
Dubal, K., Ghorpade, P., Kale, M., 2013. Studies on bioactive compounds of *Tectaria coadunata* (Wall. Ex Hook. & Grev.) C. Chr. Asian Journal of Pharmaceutical and Clinical Research 6, 186-187.
Duke, S.O., Romagni, J.G., Dayan, F.E., 2000. Natural products as sources for new mechanisms of herbicidal action. Crop Protection 19, 583-589.
Dzoyem, J.P., Aro, A.O., McGaw, L.J., Eloff, J.N., 2016. Antimycobacterial activity against different pathogens and selectivity index of fourteen medicinal plants used in southern Africa to treat tuberculosis and respiratory ailments. South African Journal of Botany 102, 70-74.
Ebone, L.A., Kovaleski, M., Deuner, C.C., 2019. Nematicides: history, mode, and mechanism action. Plant Science Today 6, 91-97.

Eguale, T., Tadesse, D., Giday, M., 2011. *In vitro* anthelmintic activity of crude extracts of five medicinal plants against egg-hatching and larval development of H*aemonchus contortus*. Journal of Ethnopharmacology 137, 108-113.

Eichenlaub, R., Gartemann, K., Burger, A., 2007. *Clavibacter michiganensis*, a group of gram-positive phytopathogenic bacteria: Plant-Associated Bacteria. Springer, Dordrecht.

El-Ansari, M.A., Aboutabl, E.A., Farrag, A.R.H., Sharaf, M., Hawas, U.W., Soliman, G.M., El-Seed, G.S., 2009. Phytochemical and pharmacological studies on *Leonotis leonurus*. Pharmaceutical Biology 47, 894-902.

Eloff, J., Masoko, P., Picard, J., 2007. Resistance of animal fungal pathogens to solvents used in bioassays. South African Journal of Botany 73, 667-669.

Eloff, J.N., 1998a. A sensitive and quick microplate method to determine the minimal inhibitory concentration of plant extracts for bacteria. Planta Medica 64, 711-713.

Eloff, J.N., 1998b. Which extractant should be used for the screening and isolation of antimicrobial components from plants? Journal of Ethnopharmacology 60, 1-8.

Eloff, J.N., 2004. Quantifying the bioactivity of plant extracts during screening and bioassay-guided fractionation. Phytomedicine 11, 370-371.

Fadipe, V.O., Mongalo, N.I., Opoku, A.R., 2015. *In vitro* evaluation of the comprehensive antimicrobial and antioxidant properties of *Curtisia dentata* (Burm.f) C.A. Sm: toxicological effect on the Human embryonic kidney (HEK293) and Human hepatocellular carcinoma (HepG2) cell lines. EXCLI journal 14, 971-983.

FAO. 2002. *Biological control in a global perspective, a view on emphesis on Duddingtonia flagrans* [Online]. [Accessed].

FAO. 2010. *Plant genetic resource for food and agriculture* [Online]. Available: www.fao.org [Accessed 3 May 2019].

FAO. 2016. *The future of food and agriculture – Trends and challenges* [Online]. [Accessed 8 April 2019].

FAO, F., 2017. The future of food and agriculture–Trends and challenges. Annual Report.

Fikreyesus, S., Kebebew, Z., Nebiyu, A., Zeleke, N., Bogale, S., 2011. Allelopathic effects of *Eucalyptus camaldulensis* Dehnh. on germination and growth of tomato. American-Eurasian Journal of Agricultural & Environmental Sciences 11, 600-608.

Finley, A., 1981. Histopathology of *Meloidogyne chitwoodi* (Golden et al.) on Russett Burbank potato. Journal of Nematology 13, 486.

Foden, W., Potter, L. 2005a. *Clausena anisata (Willd.) Hook.f. ex Benth. var. anisata. National Assessment: Red List of South African Plants version 2020.1* [Online]. [Accessed 20 September 2019].

Foden, W., Potter, L. 2005b. *Lantana rugosa Thunb. National Assessment: Red List of South African Plants version 2020.1.* [Online]. [Accessed 11 January 2020].

Foden, W., Potter, L. 2005c. *Leucosidea sericea Eckl. & Zeyh. National Assessment: Red List of South African Plants version 2020.1.* [Online]. [Accessed 10 November 2019].

Foden, W., Potter, L. 2005d. *Volkameria glabra (E.Mey.) Mabb. & Y.W.Yuan. National Assessment: Red List of South African Plants version 2020.1.* [Online]. [Accessed 22 September 2020].

Fouche, G., Sakong, B.M., Adenubi, O.T., Pauw, E., Leboho, T., Wellington, K.W., Eloff, J.N., 2016. Anthelmintic activity of acetone extracts from South African plants used on egg hatching of *Haemonchus contortus*. Onderstepoort Journal of Veterinary Research 83, 1-7.

Fouché, G., Senabe, J., Khorombi, E., Kolesnikoya, N., Maharaj, V.J., Nthambeleni, R., High throughput screening of South African plants for anti-cancer properties. Science Real and Relevant: 2nd CSIR Biennial Conference, 2008-11 2008 CSIR International Convention Centre Pretoria. 1.

Fourie, H., Spaull, V.W., Jones, R.K., Daneel, M.S., De Waele, D., 2017. Nematology in South Africa: a view from the 21st century. Springer,

Fourie, H., Zijlstra, C., McDonald, A.H., 2001. Identification of root-knot nematode species occurring in South Africa using the SCAR-PCR technique. Nematology 3, 675-680.

Fuloria, N.K., Fuloria, S., 2013. Analytical & Bioanalytical Structural Elucidation of Small Organic Molecules by 1D, 2D and Multi Dimensional-Solution NMR Spectroscopy. Journal of Analytical Bioanalytical Techniques 11, 1-8.

Gahukar, R., 2014. Potential and utilization of plant products in pest control. Integrated Pest Management. Elsevier.

Gahukar, R.T., 2018. Management of pests and diseases of important tropical/subtropical medicinal and aromatic plants: A review. Journal of Applied Research on Medicinal and Aromatic Plants 9, 1-18.

Geary, T.G., Thompson, D.P., 2001. *Caenorhabditis elegans*: how good a model for veterinary parasites? Veterinary Parasitology 101, 371-386.

Geurden, T., Chartier, C., Fanke, J., Di Regalbono, A.F., Traversa, D., von Samson-Himmelstjerna, G., Demeler, J., Vanimisetti, H.B., Bartram, D.J., Denwood, M.J., 2015. Anthelmintic resistance to ivermectin and moxidectin in gastrointestinal nematodes of cattle in Europe. International Journal for Parasitology: Drugs and Drug Resistance 5, 163-171.

Giannakou, I.O., Sidiropoulos, A., Prophetou-Athanasiadou, D., 2002. Chemical alternatives to methyl bromide for the control of root-knot nematodes in greenhouses. Pest Management Science: formerly Pesticide Science 58, 290-296.

Gomez, K.A., Gomez, A.A., 1984. Statistical procedures for agricultural research. John Wiley & Sons,

Goswami, B., Vijayalakshmi, V., 1986. Nematicidal properties of some indigenous plant materials against root-knot nematode *Meloidogyne incognita* on tomato. Indian Journal of Nematology 16, 65-68.

Govindarajan, M., 2010. Chemical composition and larvicidal activity of leaf essential oil from *Clausena anisata* (Willd.) Hook. f. ex Benth. (Rutaceae) against three mosquito species. Asian Pacific Journal of Tropical Medicine 3, 874-877.

Gundidza, M., Gweru, N., Mmbengwa, V., Ramalivhana, N., Magwa, Z., Samie, A., 2008. Phytoconstituents and biological activities of essential oil from *Rhus lancea* L. F. African Journal of Biotechnology 7, 2787-2789.

Hamza, O.J., van den Bout-van, C.J., Matee, M.I., Moshi, M.J., Mikx, F.H., Selemani, H.O., Mbwambo, Z.H., Van der Ven, A.J., Verweij, P.E., 2006. Antifungal activity of some Tanzanian plants used traditionally for the treatment of fungal infections. Journal of Ethnopharmacology 108, 124-132.

Hartwig, J., Sikora, R.A., 1991. Mode-of-action of the carbarnate nematicides cloethocarb, aldicarb and carbofuran on *Heteroderu schachtii*. Revue de Nematologie 14, 525-530.

Hatok, J., Babusikova, E., Matakova, T., Mistuna, D., Dobrota, D., Racay, P., 2009. *In vitro* assays for the evaluation of drug resistance in tumor cells. Clinical and Experimental Medicine 9, 1-7.

He, F., Lindqvist, C., Harding, W.W., 2012. Leonurenones A–C: Labdane diterpenes from *Leonotis leonurus*. Phytochemistry 83, 168-172.

Herd, R., 1996. Environmental impacts associated with endectocidal compounds. In:

Control of gastrointestinal nematodes in ruminants. EMBRAPA-CNPGL, Colonel Pacheco.

Holden-Dye, L., Walker, R. 2014. Anthelmintic drugs and nematocides: studies in *Caenorhabditis elegans*. In: Roy, PJ (ed.) WormBook: The Online Review of C. Elegans Biology.

Horak, I., Purnell, R., 2004. Parasites of domestic and wild animals in South Africa. XLV. Helminths of dairy calves on dry-land Kikuyu grass pastures in the Eastern Cape Province. Onderstepoort Journal of Veterinary Research 71, 291-306.

Horak, I.G., 2003. Parasites of domestic and wild animals in South Africa. XLII. Helminths of sheep on four farms in the Eastern Cape Province. Onderstepoot Joumal of Veterinary Research 70, 175-186.

Hough, A., Thomason, I., 1975. Effects of aldicarb on the behavior of *Heterodera schachtii* and *Meloidogyne javanica*. Journal of Nematology 7, 221.

Hounzangbe-Adote, M., Paolini, V., Fouraste, I., Moutairou, K., Hoste, H., 2005. *In vitro* effects of four tropical plants on three life-cycle stages of the parasitic nematode, *Haemonchus contortus*. Research in Veterinary Science 78, 155-160.

Hurinanthan, V., 2009.Immune modulatory effect of *Dichrostachys cinerea*, *Carpobrotus dimidiatus*, *Capparis tomentosa* and *Leonotis leonurus*. Masters Masters, Durban University of Technology.

Hussein, A.A., 2018. Chemistry of South African Lamiaceae: Structures and Biological Activity of Terpenoids. In: Perveen, S, Al-Taweel, A. (ed.) Terpenes and Terpenoids. London, United Kingdom: IntechOpen.

Hussein, H.M., Hameed, I., Ibraheem, O., 2016. Antimicrobial Activity and spectral chemical analysis of methanolic leaves extract of *Adiantum capillus*-Veneris using GC-MS and FT-IR spectroscopy. International Journal of Pharmacognosy and Phytochemical Research 8, 369-385.

Hussey, R.S., Barker, K.R., 1973. A comparison of methods of collecting inocula of *Meloidogyne* spp., including a new technique. The Plant Disease Reporter 57, 1025-1028.

Hutchings, A., Scott, A.H., Lewis, G., Cunningham, A., 1996. Zulu medicinal plants: An inventory. University of Natal press,

Ibrahim, S.K., Traboulsi, A.F., El-Haj, S., 2006. Effect of essential oils and plant extracts on hatching, migration and mortality of *Meloidogyne incognita*. Phytopathologia Mediterranea 45, 238-246.

Ignjatov, M., Milošević, D., Nikolić, Z., Gvozdanović-Varga, J., Jovičić, D., Zdjelar, G., 2012. *Fusarium oxysporum* as causal agent of tomato wilt and fruit rot. Pesticidi i Fitomedicina 27, 25-31.

Irungu, B.N., Mbabu, M.J., Kiboi, D.M., Moindi, E., Kinyua, J., Mwirichia, R.K., 2012. *In vivo* antimalarial and acute toxicity properties of hexane and chloroform extracts from *Clausena anisata* (Willd.) Benth. African Journal of Pharmacology and Therapeutics 1, 24-29.

Ito, C., Itoigawa, M., Aizawa, K., Yoshida, K., Ruangrungsi, N., Furukawa, H., 2009. γ-Lactone carbazoles from *Clausena anisata*. Journal of Natural Products 72, 1202-1204.

Ito, C., Katsuno, S., Itoigawa, M., Ruangrungsi, N., Mukainaka, T., Okuda, M., Kitagawa, Y., Tokuda, H., Nishino, H., Furukawa, H., 2000. New carbazole alkaloids from *Clausena anisata* with antitumor promoting activity. Journal of Natural Products 63, 125-128.

Iwalewa, E., McGaw, L., Naidoo, V., Eloff, J., 2007. Inflammation: The foundation of diseases and disorders. A review of phytomedicines of South African origin used to treat pain and inflammatory conditions. African Journal of Biotechnology 6, 2868-2885.

Jäger, A.K., Hutchings, A., van Staden, J., 1996. Screening of Zulu medicinal plants for prostaglandin-synthesis inhibitors. Journal of Ethnopharmacology 52, 95-100.

Jahr, H., Bahro, R., Burger, A., Ahlemeyer, J., Eichenlaub, R., 1999. Interactions between *Clavibacter michiganensis* and its host plants. Environmental Microbiology 1, 113-118.

Jimoh, F., Adedapo, A., Afolayan, A., 2010. Comparison of the nutritional value and biological activities of the acetone, methanol and water extracts of the leaves of *Solanum nigrum* and *Leonotis leonorus*. Food and Chemical Toxicology 48, 964-971.

Jones, J.T., Haegeman, A., Danchin, E.G., Gaur, H.S., Helder, J., Jones, M.G., Kikuchi, T., Manzanilla-López, R., Palomares-Rius, J.E., Wesemael, W.M., 2013. Top 10 plant-parasitic nematodes in molecular plant pathology. Molecular Plant Pathology 14, 946-961.

Jones, R.K. 2017. Nematode Control and Nematicides: Developments Since 1982 and Future Trends. In: Fourie, H, Spaull, VW, Jones, RK, , Daneel, MS & DeWaele, D (eds.) Nematology in South Africa: A View from the 21st Century. Switzerland: Springer.

Kamatou, G., Viljoen, A., Van Vuuren, S., Van Zyl, R., 2006. *In vitro* evidence of antimicrobial synergy between *Salvia chamelaeagnea* and *Leonotis leonurus*. South African Journal of Botany 72, 634-636.

Kaplan, E., Rivett, D., 1968. The structures of compounds X and Y, two labdane diterpenoids, from *Leonotis leonurus*. Journal of the Chemical Society C: Organic 262-266.

Kaplan, R.M., Vidyashankar, A.N., 2012. An inconvenient truth: global worming and anthelmintic resistance. Veterinary Parasitology 186, 70-78.

Karou, D., Nadembega, W.M., Ouattara, L., Ilboudo, D.P., Canini, A., Nikiéma, J.B., Simpore, J., Colizzi, V., Traore, A.S., 2007. African ethnopharmacology and new drug discovery. Medicinal and Aromatic Plant Science and Biotechnology 1, 61-69.

Katan, J., 2017. Diseases caused by soilborne pathogens: biology, management and challenges. Journal of Plant Pathology 99, 305-315.

Katiki, L.M., Ferreira, J.F., Zajac, A.M., Masler, C., Lindsay, D.S., Chagas, A.C.S., Amarante, A.F., 2011. *Caenorhabditis elegans* as a model to screen plant

extracts and compounds as natural anthelmintics for veterinary use. Veterinary Parasitology 182, 264-268.
Kaunda, J.S., Zhang, Y.-J., 2019. The genus *Solanum*: an ethnopharmacological, phytochemical and biological properties review. Natural Products and Bioprospecting 9, 77-137.
Kayani, M.Z., Mukhtar, T., Hussain, M.A., 2012. Evaluation of nematicidal effects of *Cannabis sativa* L. and *Zanthoxylum alatum* Roxb. against root-knot nematodes, *Meloidogyne incognita*. Crop Protection 39, 52-56.
Kearn, J., Ludlow, E., Dillon, J., O'Connor, V., Holden-Dye, L., 2014. Fluensulfone is a nematicide with a mode of action distinct from anticholinesterases and macrocyclic lactones. Pesticide Biochemistry and Physiology 109, 44-57.
Kee, N.L.A., Mnonopi, N., Davids, H., Naudé, R.J., Frost, C.L., 2008. Antithrombotic/anticoagulant and anticancer activities of selected medicinal plants from South Africa. African Journal of Biotechnology 7.
Kenechukwu, F., Mbah, C., Momoh, M., Chime, S., Umeyor, C., Ogbonna, J., 2012. Pharmacological justification for the ethnomedical use of *Clausena anisata* root-bark extract in the management of epilepsy. Journal of Applied Pharmaceutical Science 2, 036-040
Khalil, M.S., 2013. Abamectin and azadirachtin as eco-friendly promising biorational tools in integrated nematodes management programs. Journal of Plant Pathology and Microbiology 4, 2-3.
Khalil, M.S., 2014. Bright future with nematicidal phytochemicals. Biology and Medicine 6, 1.
Khan, F., Asif, M., Khan, A., Tariq, M., Ansari, T., Shariq, M., Siddiqui, M.A., 2019. Evaluation of the nematicidal potential of some botanicals against root-knot nematode, *Meloidogyne incognita* infected carrot: *In vitro* and greenhouse study. Current Plant Biology 20, 100115.
Khosa, M.C., 2013.An investigation into the potential of crude and partially separated material of selected non–crop plant species as control agents of root–knot nematodes (*Meloidogyne incognita*) in tomato. PhD Dissertation, North-West University.
Khosa, M.C., Dube, Z., De Waele, D., Daneel, M.S., 2020. Examine medicinal plants from South Africa for suppression of *Meloidogyne incognita* under glasshouse conditions. Journal of Nematology 52.
Khpalwak, W., 2012. Interaction between *Fusarium oxysporum* f. sp. *lycopersici* and *Meloidogyne incognita* in tomato. Masters, UAS, Dharwad.
Kimura, S., Sinha, N., 2008. Tomato (*Solanum lycopersicum*): a model fruit-bearing crop. Cold Spring Harbor Protocols 2008, pdb. emo105.
Knox, D.P., 2012. Developments in RNA interference and genetic transformation to define gene function in parasitic helminths. Parasitology 139, 557-559.
Koduru, S., Grierson, D.S., Afolayan, A.J., 2007. Used for treatment of cancer in the Eastern ethnobotanical information of medicinal Cape Province, South Africa. Current Science 92, 906-908.
Kokalis-Burelle, N., Rodríguez-Kàbana, R., 2006. Allelochemicals as biopesticides for management of plant-parasitic nematodes. Allelochemicals: biological control of plant pathogens and diseases. Springer.
Komansilan, A., Abadi, A.L., Yanuwiadi, B., Kaligis, D.A., 2012. Isolation and identification of biolarvicide from soursop (*Annona muricata* Linn) seeds to

mosquito (*Aedes aegypti*) larvae. nternational Journal of Engineering & Technology 12, 28-32.
Kondrollochis, M., Pain, B.F., Hague, N.G.M. 1970. The effect of phosphate and carbamoyloxime nematicides on nematodes. 10th International Symposium of Nematology. Pescara.
Kossah, R., Nsabimana, C., Zhang, H., Chen, W., 2010. Optimization of extraction of polyphenols from Syrian sumac (*Rhus coriaria* L.) and Chinese sumac (*Rhus typhina* L.) fruits. Research Journal of Phytochemistry 4, 146-153.
Krecek, R.C., Waller, P.J., 2006. Towards the implementation of the “basket of options” approach to helminth parasite control of livestock: emphasis on the tropics/subtropics. Veterinary Parasitology 139, 270-282.
Kuchta, K., Volk, R., Rauwald, H., 2013. Stachydrine in *Leonurus cardiaca*, *Leonurus japonicus*, *Leonotis leonurus*: detection and quantification by instrumental HPTLC and 1H-qNMR analyses. Die Pharmazie-An International Journal of Pharmaceutical Sciences 68, 534-540.
Kuete, V., Krusche, B., Youns, M., Voukeng, I., Fankam, A.G., Tankeo, S., Lacmata, S., Efferth, T., 2011. Cytotoxicity of some Cameroonian spices and selected medicinal plant extracts. Journal of Ethnopharmacology 134, 803-812.
Kuete, V., Tankeo, S.B., Saeed, M.E., Wiench, B., Tane, P., Efferth, T., 2014. Cytotoxicity and modes of action of five Cameroonian medicinal plants against multi-factorial drug resistance of tumor cells. Journal of Ethnopharmacology 153, 207-219.
Kumar, S., Khanna, A., 2006. Effect of neem-based products on the root-knot nematode, *Meloidogyne incognita*, and growth of tomato. Nematologia Mediterranea 34, 141-146.
Kumarasingha, R., Palombo, E.A., Bhave, M., Yeo, T., Lim, D., Tu, C., Shaw, J., Boag, P.R., 2014. Enhancing a search for traditional medicinal plants with anthelmintic action by using wild type and stress reporter *Caenorhabditis elegans* strains as screening tools. International Journal for Parasitology 44, 291-298.
Kumari, A., Baskaran, P., Van Staden, J., 2016. *In vitro* propagation and antibacterial activity in *Cotyledon orbiculata*: a valuable medicinal plant. Plant Cell, Tissue and Organ Culture (PCTOC) 124, 97-104.
Lawal, I.O., Grierson, D.S., Afolayan, A., 2015. The antibacterial activity of *Clausena anisata* hook, a South African medicinal Plant. African Journal of Traditional, Complementary and Alternative Medicines 12, 23-27.
Leela, N., Pervez, R., Ramana, K., Rosana, O., Eapen, S., 2012. Nematicidal activity of *Strychnos nuxvomica* leaf and its constituents against the root-knot nematode, *Meloidogyne incognita*. Nematologia Mediterranea 40, 157-162.
Leung, M.C., Williams, P.L., Benedetto, A., Au, C., Helmcke, K.J., Aschner, M., Meyer, J.N., 2008. *Caenorhabditis elegans*: an emerging model in biomedical and environmental toxicology. Toxicological Sciences 106, 5-28.
Li, J.-G., Dong, Y.-H., 2013. Effect of a rock dust amendment on disease severity of tomato bacterial wilt. Antonie Van Leeuwenhoek 103, 11-22.
Li, W., Zhou, J., Xu, Y., 2015. Study of the *in vitro* cytotoxicity testing of medical devices. Biomedical Reports 3, 617-620.
Mabberley, D.J., 1997. The plant-book: a portable dictionary of the vascular plants. Cambridge University Press, Cambridge, United Kingdom.
Mabona, U., Viljoen, A., Shikanga, E., Marston, A., Van Vuuren, S., 2013. Antimicrobial activity of southern African medicinal plants with dermatological

relevance: From an ethnopharmacological screening approach, to combination studies and the isolation of a bioactive compound. Journal of Ethnopharmacology 148, 45-55.
Macedo, I.T.F., Oliveira, L.M.B.d., Ribeiro, W.L.C., Santos, J.M.L.d., Silva, K.d.C., Araújo Filho, J.V.d., Camurça-Vasconcelos, A.L.F., Bevilaqua, C.M.L., 2015. Anthelmintic activity of *Cymbopogon citratus* against *Haemonchus contortus*. Revista Brasileira de Parasitologia Veterinaria 24, 268-275.
Madureira, A.M., Ramalhete, C., Mulhovo, S., Duarte, A., Ferreira, M.-J.U., 2012. Antibacterial activity of some African medicinal plants used traditionally against infectious diseases. Pharmaceutical Biology 50, 481-489.
Mafeo, T., Mashela, P., Mphosi, M., 2011. Sensitivity of selected Alliaceae seedlings to crude extracts of *Cucumis myriocarpus* fruits. African Journal of Agricultural Research 6, 158-164.
Mahwasane, S., Middleton, L., Boaduo, N., 2013. An ethnobotanical survey of indigenous knowledge on medicinal plants used by the traditional healers of the Lwamondo area, Limpopo province, South Africa. South African Journal of Botany 88, 69-75.
Makhubu, F., Khosa, M., McGaw, L., 2021. South African plants with nematicidal activity against root-knot nematodes: A review. South African Journal of Botany 139, 183-191.
Makirita, W.E., Chauka, L.J., Chacha, M., 2016. Antimicrobial and cytotoxicity activity of *Clausena anisata*, *Acokanthera shemperii* and *Olea europaea* growing in Tanzania. European Journal of Medicinal Plants 14, 1-9.
Malandrakis, A.A., Kavroulakis, N., Chrysikopoulos, C.V., 2019. Use of copper, silver and zinc nanoparticles against foliar and soil-borne plant pathogens. Science of the Total Environment 670, 292-299.
Malungane, M.M.F., 2016.Effect of crude extracts of *tulbaghia violacea* (wild garlic) on growth of tomato and supression of *Meloidogyne* species. Masters Dissertation, University of Limpopo.
Mandal, T.K., Nandi, B., 2013. Control of *Meloidogyne incognita* infestation in tomato by *Rauvolfia tetraphylla*. Indian Journal of Scientific Research 4, 61-64.
Manzanilla-López, R.H., Starr, J.L., 2009. 10 Interactions with Other Pathogens. In: Perry, RN, Moens, M & Starr, JL (eds.) Root-knot nematodes. CABI.
Maphosa, V., Adedapo, A., Moyo, B., Masika, P., 2012. Anti-inflammatory and analgesic activities of the aqueous extract of *Leonotis leonurus* leaves in rats. African Journal of Biotechnology 11, 6878-6883.
Maphosa, V., Masika, P., Adedapo, A., 2008. Safety evaluation of the aqueous extract of *Leonotis leonurus* shoots in rats. Human & Experimental Toxicology 27, 837-843.
Maphosa, V., Masika, P.J., 2010. Ethnoveterinary uses of medicinal plants: A survey of plants used in the ethnoveterinary control of gastro-intestinal parasites of goats in the Eastern Cape Province, South Africa. Pharmaceutical Biology 48, 697-702.
Maphosa, V., Masika, P.J., Bizimenyera, E.S., Eloff, J., 2010. *In-vitro* anthelminthic activity of crude aqueous extracts of *Aloe ferox*, *Leonotis leonurus* and *Elephantorrhiza elephantina* against *Haemonchus contortus*. Tropical Animal Health and Production 42, 301-307.

Maroyi, A., Mosina, G.K., 2014. Medicinal plants and traditional practices in peri-urban domestic gardens of the Limpopo province, South Africa. Indian Journal of Traditional Knowledge 13.

Martin, R.J., Purcell, J., Robertson, A., Valkanov, M., 2002. Neuromuscular Organisation and Control in Nematodes. In: Lee, DL (ed.) The Biology of Nematodes. London, New York: CRC Press.

Masafu, M.M., Mbajiorgu, C., Nemadodzi, L., Kabine, E., 2016. A study of natural habitats and uses of medicinal plants in Thulamela and JS Moroka Municipalities, South Africa. Indian Journal of Traditional Knowledge 15, 363-369.

Masevhe, N.A., Awouafack, M.D., Ahmed, A.S., McGaw, L.J., Eloff, J.N., 2013. Clerodendrumic acid, a new triterpenoid from *Clerodendrum glabrum* (Verbenaceae), and antimicrobial activities of fractions and constituents. Helvetica Chimica Acta 96, 1693-1703.

Masevhe, N.A., McGaw, L.J., Eloff, J.N., 2015. The traditional use of plants to manage candidiasis and related infections in Venda, South Africa. Journal of Ethnopharmacology 168, 364-372.

Mashela, P., 2002. Ground wild cucumber fruits suppress numbers of *Meloidogyne incognita* on tomato in microplots. Nematropica 13-20.

Mashela, P., Nthangeni, M., 2002. Efficacy of *Ricinus communis* fruit meal with and without Bacillus species on suppression of *Meloidogyne incognita* and growth of tomato. Journal of Phytopathology 150, 399-402.

Mashela, P.W., De Waele, D., Dube, Z., Khosa, M.C., Pofu, K.M., Tefu, G., Daneel, M.S., Fourie, H. 2017. Alternative Nematode Management Strategies. In: Fourie, H, Spaull, VW, Jones, RK, Daneel, MS & De Waele, D (eds.) Nematology in South Africa: A View from the 21st Century. Switzerland: Springer.

Mashela, P.W., Dube, Z.P., Pofu, K.M., 2015. Managing the phytotoxicity and inconsistent nematode suppression in soil amended with phytonematicides. Organic amendments and soil suppressiveness in plant disease management. Springer.

Masika, P., Van Averbeke, W., Sonandi, A., 2000. Use of herbal remedies by small-scale farmers to treat livestock diseases in central Eastern Cape Province, South Africa. Journal of the South African Veterinary Association 71, 87-91.

Masoko, P., Eloff, J., 2006. Bioautography indicates the multiplicity of antifungal compounds from twenty-four southern African Combretum species (Combretaceae). African Journal of Biotechnology 5, 1625-1647.

Matthiessen, J.N., Kirkegaard, J.A., 2006. Biofumigation and Enhanced Biodegradation: Opportunity and Challenge in Soilborne Pest and Disease Management. Critical Reviews in Plant Sciences 25, 235-265.

Mavundza, E., Mhlongo, S., Cebekhulu, S., Street, R., Bissessur, A., Sithole, Z., Baijnath, H., 2018. Phytochemical screening of four South African poisonous medicinal plants of the family Euphorbiaceae. South African Journal of Botany 115, 322.

Max, R., 2010. Effect of repeated wattle tannin drenches on worm burdens, faecal egg counts and egg hatchability during naturally acquired nematode infections in sheep and goats. Veterinary Parasitology 169, 138-143.

Mayekiso, K., Eloff, J., McGaw, L., 2009. Screening of South African plants for antibacterial and antimycobacterial activity. African Journal of Traditional, Complementary and Alternative Medicines 6, 319-319.
McDonald, A., Nicol, J., 2005. Plant parasitic nematodes in subtropical and tropical agriculture. In: Luc, M, Sikora, RA & Bridge, J (eds.) Nematode parasites of cereals. 2 ed. UK,Wallingford: CABI.
McFadden, J. 25 February 2021 2021. You say Tomato's I say Tomatoes…. *Sandy Ridge Vineyards and Mercantile* [Online]. Available from: www.sandyridgevineyards.com [Accessed 20 March 2021].
McGaw, L., Eloff, J., 2005. Screening of 16 poisonous plants for antibacterial, anthelmintic and cytotoxic activity *in vitro*. South African Journal of Botany 71, 302-306.
McGaw, L.J., Eloff, J.N., 2008. Ethnoveterinary use of southern African plants and scientific evaluation of their medicinal properties. Journal of Ethnopharmacology 119, 559-574.
McGaw, L.J., Jager, A.K., van Staden, J., 2000. Antibacterial, anthelmintic and anti-amoebic activity in South African medicinal plants. Journal of Ethnopharmacology 72, 247-263.
McGaw, L.J., Van der Merwe, D., Eloff, J., 2007. In vitro anthelmintic, antibacterial and cytotoxic effects of extracts from plants used in South African ethnoveterinary medicine. The Veterinary Journal 173, 366-372.
McKenzie, J., Green, I., Mugabo, P., 2006. Leonurun, a novel labdane diterpenoid from *Leonotis leonurus*. South African Journal of Chemistry 59, 114–116-114–116.
McSorley, R., Gallaher, R., 1995. Cultural practices improve crop tolerance to nematodes. Nematropica 53-60.
McSorley, R., Wang, K.-H., Church, G., 2008. Suppression of root-knot nematodes in natural and agricultural soils. Applied Soil Ecology 39, 291-298.
Mera, I., Falconí, D.E.G., Córdova, V.M., 2019. Secondary metabolites in plants: main classes, phytochemical analysis and pharmacological activities. Rev Bionatura 4, 1000-9.
Mitkowski, N., Abawi, G. 2003. Root-knot nematodes. The Plant Health Instructor. DOI: 10.1094. PHI-I-2003-0917-01.
Mnonopi, N., Levendal, R.-A., Davies-Coleman, M.T., Frost, C.L., 2011. The cardioprotective effects of marrubiin, a diterpenoid found in *Leonotis leonurus* extracts. Journal of Ethnopharmacology 138, 67-75.
Mnonopi, N., Levendal, R.-A., Mzilikazi, N., Frost, C., 2012. Marrubiin, a constituent of *Leonotis leonurus*, alleviates diabetic symptoms. Phytomedicine 19, 488-493.
Moens, M., Perry, R.N., Starr, J.L., 2009. *Meloidogyne* species–a diverse group of novel and important plant parasites. Root-knot nematodes 1, 483.
Mosmann, T., 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. Journal of Immunological Methods 65, 55-63.
Motsei, M.L., Lindsey, K.v., Van Staden, J., Jäger, A., 2003. Screening of traditionally used South African plants for antifungal activity against *Candida albicans*. Journal of Ethnopharmacology 86, 235-241.
Muhizi, T., 2002.The extraction, purification and evaluation of compounds from the leaves of *Leonotis Leonorus* for anticonvulsant activity. Masters, University of the Western Cape.

Mukandiwa, L., Ahmed, A., Eloff, J.N., Naidoo, V., 2013. Isolation of seselin from *Clausena anisata* (Rutaceae) leaves and its effects on the feeding and development of *Lucilia cuprina* larvae may explain its use in ethnoveterinary medicine. Journal of Ethnopharmacology 150, 886-891.

Mukandiwa, L., Eloff, J.N., Naidoo, V., 2016. Repellent and mosquitocidal effects of leaf extracts of *Clausena anisata* against the *Aedes aegypti* mosquito (Diptera: Culicidae). Environmental Science and Pollution Research 23, 11257-11266.

Mulubwe, N., 2007.A comparative study of the phosphodiesterase 4 inhibitory activity of *Artemisia afra*, *Leonotis leonorus* and *Mentha longifolia*. Masters Dissertation, University of the Western Cape.

Munodawafa, T., Chagonda, L.S., Moyo, S.R., 2013. Antimicrobial and phytochemical screening of some Zimbabwean medicinal plants. Journal of Biologically Active Products from Nature 3, 323-330.

Murray, L., Geldhof, P., Clark, D., Knox, D.P., Britton, C., 2007. Expression and purification of an active cysteine protease of *Haemonchus contortus* using *Caenorhabditis elegans*. International Journal for Parasitology 37, 1117-1125.

Naidoo, D., Maharaj, V., Crouch, N., Ngwane, A., 2011. New labdane-type diterpenoids from *Leonotis leonurus* support circumscription of Lamiaceae s.l. Biochemical Systematics and Ecology 39, 216–219.

Nair, A.R., Kotiyal, J., Bhardwaj, D., 1983. Myricetin 7, 4′-dimethyl ether and its 3-galactoside from *Rhus lancea*. Phytochemistry 22, 318-319.

Nair, J., Aremu, A., Van Staden, J., 2012. Anti-inflammatory effects of *Leucosidea sericea* (Rosaceae) and identification of the active constituents. South African Journal of Botany 80, 75-76.

Natarajan, N., Cork, A., Boomathi, N., Pandi, R., Velavan, S., Dhakshnamoorthy, G., 2006. Cold aqueous extracts of *African marigold*, *Tagetes erecta* for control tomato root knot nematode, *Meloidogyne incognita*. Crop Protection 25, 1210-1213.

Ndlovu, G., Fouche, G., Tselanyane, M., Cordier, W., Steenkamp, V., 2013. *In vitro* determination of the anti-aging potential of four southern African medicinal plants. BMC Complementary and Alternative Medicine 13, 1-7.

Ndomo, A.F., Ngamo, L.T., Tapondjou, L.A., Tchouanguep, F.M., Hance, T., 2008. Insecticidal effects of the powdery formulation based on clay and essential oil from the leaves of C*lausena anisata* (Willd.) JD Hook ex. Benth. (Rutaceae) against *Acanthoscelides obtectus* (Say)(Coleoptera: Bruchidae). Journal of Pest Science 81, 227.

Ngadjui, B.T., Ayafor, J.F., Sondengam, B.L., Connolly, J.D., 1989. Prenylated coumarins from the leaves of *Clausena anisata*. Journal of Natural Products 52, 243-247.

Ngassoum, M.B., Jirovetz, L., Buchbauer, G., Schmaus, G., Hammerschmidt, F.-J., 1999. Chemical composition and olfactory evaluation of the essential oils of leaves and seeds of *Clausena anisata* (Willd.) JD Hook. ex. Benth. from Cameroon. Journal of Essential Oil Research 11, 231-237.

Ngegba, M., Sesay, M., Bangura, T., 2016. The impact of vegetable farming on the livelihood of small-scale farmers in Koinadugu District Northern Sierra Leone. Glob. International Journal of Bio-Science and Bio-Technology 5, 42-49.

Ngobeni, A., 2012.Isolation and characterization of bio-active compounds from *Euphorbia inaequilatera* and *Dicerocaryum senecioides*. Masters Dissertation, University of Limpopo (Turfloop Campus).

Nguyen, L.T.T., Jang, J.Y., Kim, T.Y., Yu, N.H., Park, A.R., Lee, S., Bae, C.-H., Yeo, J.H., Hur, J.-S., Park, H.W., 2018. Nematicidal activity of verrucarin A and roridin A isolated from *Myrothecium verrucaria* against *Meloidogyne incognita*. Pesticide Biochemistry and Physiology 148, 133-143.

Nguyen, L.T.T., Seo, D.-J., Kim, K.-Y., Park, R.-D., Kim, D.-H., Han, Y.-S., Kim, T.-H., Jung, W.-J., 2013. Nematicidal activity of 3, 4-dihydroxybenzoic acid purified from *Terminalia nigrovenulosa* bark against *Meloidogyne incognita*. Microbial Pathogenesis 59, 52-59.

Nicol, J., Turner, S., Coyne, D., den Nijs, L., Hockland, S., Maafi, Z.T., 2011. Current nematode threats to world agriculture. In: Jones, J, Gheysen, G & Fenoll, C (eds.) Genomics and molecular genetics of plant-nematode interactions. Heidelberg, Germany: Springer.

Nielsen, N.D., Sandager, M., Stafford, G.I., van Staden, J., Jäger, A.K., 2004. Screening of indigenous plants from South Africa for affinity to the serotonin reuptake transport protein. Journal of Ethnopharmacology 94, 159-163.

Noling, J. 1997 Movement and toxicity of nematicides in the plant root zone. [Accessed 12 August 2019].

Nsuala, B.N., Enslin, G., Viljoen, A., 2015. “Wild cannabis”: A review of the traditional use and phytochemistry of *Leonotis leonurus*. Journal of Ethnopharmacology 174, 520-539.

Nukenine, E.N., Adler, C., Reichmuth, C., 2010. Efficacy of *Clausena anisata* and *Plectranthus glandulosus* leaf powder against *Prostephanus truncatus* (Coleoptera: Bostrichidae) and two strains of *Sitophilus zeamais* (Coleoptera: Curculionidae) on maize. Journal of Pest Science 83, 181-190.

Obi, C., Potgieter, N., Bessong, P., Masebe, T., Mathebula, H., Molobela, P., Jäger, A., 2003. *In vitro* antibacterial activity of Venda medicinal plants. South African Journal of Botany 69, 199-203.

Obikeze, K., 2004.Cardiovascular effects of *Leonotis leonurus* extracts in normotensive rats and in isolated perfused rat heart. Masters Dissertation, University of the Western Cape.

Obikeze, K.C., McKenzie, J.M., Green, I.R., Mugabo, P., 2008. Characterization and cardiovascular effects of (13S)-9α, 13α-epoxylabda-6β (19), 15 (14) diol dilactone, a diterpenoid isolated from *Leonotis leonurus*. South African Journal of Chemistry 61, 119-122.

Obradovic, A., Jones, J., Balogh, B., Momol, M., 2008. Integrated management of tomato bacterial spot. Integrated management of diseases caused by fungi, phytoplasma and bacteria. Springer.

Ogumo, E., 2014.Use of eco-friendly strategies in suppression of root-knot nematodes in French bean (*Phaseolus vulgaris*) in Kenya. Masters, University of Nairobi.

Ojewole, J., 2005. Antinociceptive, antiinflammatory and antidiabetic effects of *Leonotis leonurus* (L.) R. BR. (Lamiaceae) leaf aqueous extract in mice and rats. Methods and Findings in Experimental and Clinical Pharmacology 27, 257-264.

Ojewole, J.A., 2002. Hypoglycaemic effect of *Clausena anisata* (Willd) Hook methanolic root extract in rats. Journal of Ethnopharmacology 81, 231-237.

Oka, Y., 2010. Mechanisms of nematode suppression by organic soil amendments—a review. Applied Soil Ecology 44, 101-115.

Oka, Y., 2012. Nematicidal activity of *Verbesina encelioides* against the root-knot nematode *Meloidogyne javanica* and effects on plant growth. Plant and Soil 355, 311-322.

Okeke, C.U., Iroka, C.F., Okereke, N.C., Nyanayo, B.L., 2015. Effects of the crude water extract of the leaves of *Morinda lucida* on the germination and growth of *Amaranthus spinosus* and *Amaranthus hybridus*. European Journal of Medicinal Plants 9, 1-9.

Okokon, J.E., Etebong, E.O., Udobang, J.A., Essien, G.E., 2012. Antiplasmodial and analgesic activities of *Clausena anisata*. Asian Pacific Journal of Tropical Medicine 5, 214-219.

Olaniyi, M.O., 2015. The use of botanicals in the management of plant parasitic nematodes. The International Journal of Science and Technoledge 3, 300.

Olaokun, O., Mkolo, N., Mogale, M., King, P., 2017. Phytochemical Screening, Antioxidant, Anti-inflammatory and Glucose Utilization Activities of Three South African Plants Used Traditionally to Treat Diseases. Biology and Medicine 9, 2.

Olorunnisola, O., Bradley, G., Afolayan, A., 2012. Antioxidant activity of acetone and ethanolic leaves extracts of *Hippobromus pauciflorus* (Lf) Radlk. African Journal of Biotechnology 11, 1206-1213.

Ondua, M., Adebayo, S., Shai, L., Lebelo, S., 2016. The anti-inflammatory and anti-nociceptive activities of some medicinal plant species used to treat inflammatory pain conditions in southern Africa. International Journal of Pharmacognosy and Phytochemical Research 8, 1571-1575.

Onkendi, E.M., Kariuki, G.M., Marais, M., Moleleki, L.N., 2014. The threat of root-knot nematodes (*Meloidogyne* spp.) in Africa: A review. Plant Pathology 63, 727-737.

Osei, K., Moss, R., Nafeo, A., Addico, R., Agyemang, A., Danso, Y., Asante, J., 2011. Management of plant parasitic nematodes with antagonistic plants in the forest-savanna transitional zone of Ghana. Journal of Applied Biosciences 37, 2491-2495.

Oyedemi, S., Afolayan, A., 2011. *In vitro* and *in vivo a*ntioxidant activity of aqueous leaves extract of *Leonotis leonurus* (L.) R. Br. International Journal of Pharmacology 7, 248-256.

Pallant, J. 2001. SPSS Survival Manual: A Step by Step Guide to Data Analysis Using SPSS. Australia: Crows Nest, Allen and Unwin.

Pavaraj, M., Bakavathiappan, G., Baskaran, S., 2012. Evaluation of some plant extracts for their nematicidal properties against root-knot nematode, *Meloidogyne incognita*. Journal of Biopesticides 5, 106.

Pavela, R., Maggi, F., Lupidi, G., Mbuntcha, H., Woguem, V., Womeni, H.M., Barboni, L., Tapondjou, L.A., Benelli, G., 2018. *Clausena anisata* and *Dysphania ambrosioides* essential oils: from ethno-medicine to modern uses as effective insecticides. Environmental Science and Pollution Research 25, 10493-10503.

Pendota, S., Yakubu, M., Grierson, D., Afolayan, A., 2009. Anti-inflammatory, analgesic and antipyretic activities of the aqueous extract of *Hippobromus pauciflorus* (Lf) Radlk leaves in male Wistar rats. African Journal of Biotechnology 8.

Perry, B.D., Randolph, T.F., 1999. Improving the assessment of the economic impact of parasitic diseases and of their control in production animals. Veterinary Parasitology 84, 145-168.

Perry, E.J., Ploeg, A.T. 2010. Integrated pest management for home gardeners and landscape professionals. In: Fayard, ML (ed.). California: University of California.

Pitan, O.O., Ayelaagbe, O., Wang, H.L., Wang, C.Z., 2009. Identification, isolation and characterization of the antifeedant constituent of *Clausena anisata* against *Helicoverpa armigera* (Lepidoptera: Noctuidae). Insect Science 16, 247-253.

Pitso, T.R., Ashafa, A.O., 2015. Influence of different drying methods on the composition and antimicrobial activities of essential oil from *Leucosidea sericea* Eckl. & Zeyh. Journal of Essential Oil Bearing Plants 18, 146-153.

Pope, C.N., 1999. Organophosphorus pesticides: do they all have the same mechanism of toxicity? Journal of Toxicology and Environmental Health Part B: Critical Reviews 2, 161-181.

Pretorius, J., Magama, S., Zietsman, P., van Wyk, B.-E., 2003. Growth inhibition of plant pathogenic bacteria and fungi by extracts from selected South African plant species. South African Journal of Botany 69, 186-192.

Prior, P., Ailloud, F., Dalsing, B.L., Remenant, B., Sanchez, B., Allen, C., 2016. Genomic and proteomic evidence supporting the division of the plant pathogen *Ralstonia solanacearum* into three species. BMC Genomics 17, 90.

Rasoanaivo, P., Ratsimamanga-Urverg, S., 1993. Biological evaluation of plants with reference to the Malagasy flora. Napreca, Madagascar.

Rayne, S., Mazza, G., 2007. Biological activities of extracts from sumac *(Rhus* spp.): a review. Nature Precedings 1-1.

Reddy, D.D.R., Seshadri, A.R., 1971. Studies on some systemic nematicides. 11. Further studies on the action of thionazin on *Meloidogyne incognita* and *Rotylenchulus reniformis*. Indian Journal Nematology 2, 182-190.

Reddy, S.A., Bagyaraj, D., Kale, R.D., 2012. Management of tomato bacterial spot caused by *Xanthomonas campestris* using vermicompost. Journal of Biopesticides 5, 10.

Renčo, M., Sasanelli, N., Maistrello, L., 2014. Plants as natural sources of nematicides. In: Davis, LM (ed.) Nematodes: Comparative Genomics, Disease Management and Ecological Importance. New York: NOVA Science.

Riddle, D.L., Blumenthal, T., Meyer, B.J., Priess, J.R., 1997. *C. elegans* II, Monograph 33. Cold Spring Harbor Laboratory Press, New York.

Risa, J., Risa, A., Adsersen, A., Gauguin, B., Stafford, G.I., van Staden, J., Jäger, A.K., 2004. Screening of plants used in southern Africa for epilepsy and convulsions in the GABAA-benzodiazepine receptor assay. Journal of Ethnopharmacology 93, 177-182.

Ritter, R.A., Monteiro, M.V.B., Monteiro, F.O.B., Rodrigues, S.T., Soares, M.L., Silva, J.C.R., Palha, M.d.D.C., Biondi, G.F., Rahal, S.C., Tourinho, M.M., 2012. Ethnoveterinary knowledge and practices at Colares island, Pará state, eastern Amazon, Brazil. Journal of Ethnopharmacology 144, 346-352.

Roeber, F., Jex, A.R., Gasser, R.B., 2013. Impact of gastrointestinal parasitic nematodes of sheep, and the role of advanced molecular tools for exploring epidemiology and drug resistance-an Australian perspective. Parasites & Vectors 6, 153.

Saad, B., Azaizeh, H., Abu-Hijleh, G., Said, O., 2006. Safety of traditional Arab herbal medicine. Evidence-Based Complementary and Alternative Medicine 3, 433-439.

Sahebani, N., Hadavi, N., 2008. Biological control of the root-knot nematode *Meloidogyne javanica* by *Trichoderma harzianum*. Soil Biology and Biochemistry 40, 2016-2020.
Samie, A., Nefefe, T., 2012. Antifungal activities of essential oils from Southern African medicinal plants against five *Fusarium* species. Journal of Medicinal Plants Research 6, 465-478.
Samie, A., Nefefe, T., Gundidza, M., Mmbengwa, V., Magwa, M., 2012. Antimicrobial activities of essential oils from Southern Africa against selected bacterial and fungal organisms. African Journal of Biotechnology 11, 15560-15568.
SANBI. 2018. *Leonotis leonurus* [Online]. Available: http://pza.sanbi.org/ [Accessed 23 November 2018].
SANBI. 2019a. *Cotyledon orbiculata L.* [Online]. [Accessed 11 November 2018].
SANBI. 2019b. *Lantana rugosa Thunb.* [Online]. Available: http://pza.sanbi.org [Accessed 15 September 2019].
Sánchez-Bayo, F., 2011. Impacts of agricultural pesticides on terrestrial ecosystems. Ecological impacts of toxic chemicals 2011, 63-87.
Santos, B.M., Gilreath, J.P., Motis, T.N., Noling, J.W., Jones, J.P., Norton, J.A., 2006. Comparing methyl bromide alternatives for soilborne disease, nematode and weed management in fresh market tomato. Crop Protection 25, 690-695.
Sasser, J., Eisenback, J., Carter, C., Triantaphyllou, A., 1983. The international *Meloidogyne* project-its goals and accomplishments. Annual Review of Phytopathology 21, 271-288.
Sawleha, Q., Dixit, A., Dixit, P., 2010. Use of medicinal plants to control *Haemonchus contortus* infection in small ruminants. Veterinary World 3, 515.
Schoenian, S. 2003. *Integrated parasite management. Maryland Cooperative extension. Maryland Small Ruminant* [Online]. University of Maryland Extension Small Ruminant Program. Available: www.sheepandgoat. com/articles/IPM.html. [Accessed 14 August 2019].
Seid, A., Fininsa, C., Mekete, T., Decraemer, W., Wesemael, W.M., 2015. Tomato (*Solanum lycopersicum*) and root-knot nematodes (*Meloidogyne* spp.)–a century-old battle. Nematology 17, 995-1009.
Senthilkumar, A., Venkatesalu, V., 2009. Phytochemical analysis and antibacterial activity of the essential oil of *Clausena anisata* (Willd.) Hook. f. ex Benth. International Journal of Integrative Biology 5, 116-120.
Seshathri, K., 2012. Antimicrobial properties of Ethiopian chewing sticks against *Candida albicans*. Journal of Applied Pharmaceutical Science 2, 45.
Shai, L., McGaw, L., Masoko, P., Eloff, J., 2008. Antifungal and antibacterial activity of seven traditionally used South African plant species active against *Candida albicans*. South African Journal of Botany 74, 677-684.
Shai, L.J., Bizimenyera, E., Bagla, V., McGaw, L.J., Eloff, J.N., 2009. *Curtisia dentata* (Cornaceae) leaf extracts and isolated compounds inhibit motility of parasitic and free-living nematodes. Onderstepoort Journal of Veterinary Research 76, 249-256.
Sharma, R., Kishore, N., Hussein, A., Lall, N., 2014. The potential of *Leucosidea sericea* against *Propionibacterium acnes*. Phytochemistry Letters 7, 124-129.
Sichilongo, K.F., Obuseng, V.C., Okatch, H., 2012. Applications of Gas Chromatography–Mass Spectrometry (GC–MS): An Examination of Selected African Cases. Chromatographia 75, 1017-1037.

Silvestre, A., Cabaret, J., 2004. Nematode parasites of animals are more prone to develop xenobiotic resistance than nematode parasites of plants. Parasite 11, 119-129.
Simon, M., Ajanusi, O., Abubakar, M., Idris, A., Suleiman, M., 2012. The anthelmintic effect of aqueous methanol extract of *Combretum molle* (R. Br. x. G. Don)(Combretaceae) in lambs experimentally infected with *Haemonchus contortus*. Veterinary Parasitology 187, 280-284.
Simpkin, K.G., Coles, G.C., 1981. The use of *Caenorhabditis elegans* for anthelmintic screening. Journal of Chemical Technology and Biotechnology 31, 66-69.
Singh, N., Siddiqui, Z.A., 2012. Inoculation of Tomato with *Ralstonia solanacearum, Xanthomonas campestris,* and *Meloidogyne javanica*. International Journal of Vegetable Science 18, 78-86.
Sivakumar, M., Gunasekaran, K., 2011. Management of root knot nematodes in tomato, chilli and brinjal by neem oil formulations. Journal of Biopesticides 4, 198.
Skantar, A.M., Agama, K., Meyer, S.L., Carta, L.K., Vinyard, B.T., 2005. Effects of geldanamycin on hatching and juvenile motility in *Caenorhabditis elegans* and *Heterodera glycines*. Journal of Chemical Ecology 31, 2481-2491.
Smith, A., 1895. A contribution to South African materia medica: Chiefly from plants in use among the natives. JC Juta & Co, South Africa, Cape Town.
Soler, R., Nicolás, J., Caballero, S., Yubero, E., Crespo, J., 2016. Depletion of tropospheric ozone associated with mineral dust outbreaks. Environmental Science and Pollution Research 23, 19376-19386.
Songue, J.L., Dongo, E., Mpondo, T.N., White, R.L., 2012. Chemical constituents from stem bark and roots of *Clausena anisata*. Molecules 17, 13673-13686.
Soulsby, E.J., 1986. Helminths, arthropods and protozoa of domesticated animals. Balliere, Tindall, London.
Soyelu, O., Masika, P., 2009. Traditional remedies used for the treatment of cattle wounds and myiasis in Amatola Basin, Eastern Cape Province, South Africa. Onderstepoort Journal of Veterinary Research 76, 393-397.
Soyuncu, S., Oktay, C., Berk, Y., Eken, C., 2007. Abamectin intoxication with coma and hypotension. Clinical Toxicology 45, 299-300.
Spiegler, V., Liebau, E., Hensel, A., 2017. Medicinal plant extracts and plant-derived polyphenols with anthelmintic activity against intestinal nematodes. Natural Product Reports 34, 627-643.
Spurr Jr, H., 1985. Mode of action of nematicides. In: Barker, KR, Carter, CC & Sasser, JN (eds.) An advanced treatise on *Meloidogyne*, vol. 1, Biology and control. Raleigh: North Carolina Department of Plant Pathology, North Carolina State University.
Stafford, G., Jäger, A., Van Staden, J., 2005. Effect of storage on the chemical composition and biological activity of several popular South African medicinal plants. Journal of Ethnopharmacology 97, 107-115.
Steenkamp, V., 2003. Traditional herbal remedies used by South African women for gynaecological complaints. Journal of Ethnopharmacology 86, 97-108.
Steenkamp, V., Mathivha, E., Gouws, M., Van Rensburg, C., 2004. Studies on antibacterial, antioxidant and fibroblast growth stimulation of wound healing remedies from South Africa. Journal of Ethnopharmacology 95, 353-357.
Steyn, P.S., van Heerden, F.R., Vleggaar, R., Anderson, L.A., 1986. Bufadienolide glycosides of the Crassulaceae. Structure and stereochemistry of orbicusides

A—C, novel toxic metabolites of *Cotyledon orbiculata*. Journal of the Chemical Society, Perkin Transactions 1, 1633-1636.
Stiernagle, T. 2006. *Maintenance of C. elegans* [Online]. [Accessed 15 October 2018].
Stoffersen, A., Winstrup, M., Nieminen, R., Allerton, T. 2011. Medicinal Plants and Traditional Healing in Contemporary Rural South Africa. The sustainability of medicinal plant use in the local culture in Ongeluksnek, Eastern Cape, South Africa. University of Copenhagen.
Strajnar, P., Sirca, S., 2011. The effect of some insecticides, natural compounds and tomato cv. Venezia with Mi gene on the nematode *Meloidogyne ethiopica* (Nematoda) reproduction. Acta Agriculturae Slovenica 97, 5.
Subapriya, R., Nagini, S., 2005. Medicinal properties of neem leaves: a review. Current Medicinal Chemistry-Anti-Cancer Agents 5, 149-156.
Suliman, A., 2010.The antimicrobial activity and chemical profile of traditional medicinal plants indigenous to Southern Africa used to treat respiratory tract infections. Masters Dissertation, University of the Witwatersrand.
Tabassum, S., Ahmed, M., Mirza, B., Naeem, M., Zia, M., Shanwari, Z.K., Khan, G.M., 2017. Appraisal of phytochemical and *in vitro* biological attributes of an unexplored folklore: *Rhus punjabensis* stewart. BMC Complementary and Alternative Medicine 17, 1-13.
Tadesse, D., Eguale, T., Giday, M., Mussa, A., 2009. Ovicidal and larvicidal activity of crude extracts of *Maesa lanceolata* and *Plectranthus punctatus* against *Haemonchus contortus*. Journal of Ethnopharmacology 122, 240-244.
Takaishi, K., Izumi, M., Baba, N., Kawazu, K., Nakajima, S., 2008. Synthesis and biological evaluation of alkoxycoumarins as novel nematicidal constituents. Bioorganic & Medicinal Chemistry Letters 18, 5614-5617.
Tankeo, S.B., Tane, P., Kuete, V., 2015. *In vitro* antibacterial and antibiotic-potentiation activities of the methanol extracts from *Beilschmiedia acuta*, *Clausena anisata*, *Newbouldia laevis* and *Polyscias fulva* against multidrug-resistant Gram-negative bacteria. BMC Complementary and Alternative Medicine 15, 412.
Tariq, K.A., 2018. Use of plant anthelmintics as an alternative control of helminthic infections in sheep. Res J Zool 1.
Taylor, M., Coop, R., Wall, R., 2007. Veterinary Epidemiology. Black well, Asia.
Terblanche, M., Adelaar, T., 1965. A note on the toxicity of *Cotyledon orbiculata* L. Journal of the South African Veterinary Association 36, 555-559.
Thompson, D., Klein, R., Geary, T., 1996. Prospects for rational approaches to anthelmintic discovery. Parasitology 113, S217-S238.
Thovhakale, L.P., van Der Westhuizen, C., Mashela, P.W., 2006. Responses of root-knot nematode densities to aqueous extracts of Chilli and Tamboti. Agriprobe 3, 10-12.
TNAU. 2015. *Introduction to Sapota Cultivation* [Online]. [Accessed].
Tranier, M.-S., Pognant-Gros, J., Quiroz, R.D.l.C., González, C.N.A., Mateille, T., Roussos, S., 2014. Commercial biological control agents targeted against plant-parasitic root-knot nematodes. Brazilian Archives of Biology and Technology 57, 831-841.
Tripathi, A.K., Bhakuni, R.S., Upadhyay, S., Gaur, R., 2011. Insect feeding deterrent and growth inhibitory activities of scopoletin isolated from *Artemisia annua* against *Spilarctia obliqua* (Lepidoptera: Noctuidae). Insect Science 18, 189-194.

Tsay, T., Wu, S., Lin, Y., 2004. Evaluation of Asteraceae plants for control of *Meloidogyne incognita*. Journal of Nematology 36, 36.
van Wyk, B.-E., De Wet, H., van Heerden, F., 2008. An ethnobotanical survey of medicinal plants in the southeastern Karoo, South Africa. South African Journal of Botany 74, 696-704.
van Wyk, B.-E., Oudtshoorn, B.v., Gericke, N., 1997. Medicinal Plants of South Africa. Briza, Pretoria, South Africa.
van Wyk, B.-E., van Ousdtshoorn, B., Gericke, N., 2002. Medicinal plants of South Africa. Briza, Pretoria, South Africa
van Wyk, B., Gericke, N., 2000. Peoples Plants. A Guide to Useful of Southern Africa. Briza Pretoria, South Africa.
van Wyk, B.E., Oudtshoorn, B.V., Gericke, N., 2009. Medicinal plants of South Africa. Briza, Pretoria, South Africa.
Vijayabaskar, G., Elango, V., 2018. Determination of phytocompounds in *Withania somnifera* and *Smilax china* using GC-MS. Journal of Pharmacognosy and Phytochemistry 7, 554-557.
Wahba, H.M., AbouZid, S.F., Sleem, A.A., Apers, S., Pieters, L., Shahat, A.A., 2011. Chemical and biological investigation of some *Clerodendrum* species cultivated in Egypt. Pharmaceutical Biology 49, 66-72.
Waller, P., Chandrawathani, P., 2005. *Haemonchus contortus*: parasite problem No. 1 from tropics-Polar Circle. Problems and prospects for control based on epidemiology. Tropical Biomedicine 22, 131-137.
Wanas, A., Khalil, H., Fouad, M., Kamel, M., Radwan, M., ElSohaly, M., 2013. Phytochemical Study of the Leaves of *Clerodendrum glabrum* (Verbenaceae). Planta Medica 79, PN76.
Waterman, C., Smith, R.A., Pontiggia, L., DerMarderosian, A., 2010. Anthelmintic screening of Sub-Saharan African plants used in traditional medicine. Journal of Ethnopharmacology 127, 755-759.
Watt, J.M., Breyer-Brandwijk, M.G., 1962a. The Medicinal and Poisonous Plants of Southern and Eastern Africa being an Account of their Medicinal and other Uses, Chemical Composition, Pharmacological Effects and Toxicology in Man and Animal. E. & S. Livingstone Ltd., Teviot Place, Edinburgh.
Watt, J.M., Breyer-Brandwijk, M.G., 1962b. The Medicinal and Poisonous Plants of Southern and Eastern Africa being an Account of their Medicinal and other Uses, Chemical Composition, Pharmacological Effects and Toxicology in Man and Animal. The Medicinal and Poisonous Plants of Southern and Eastern Africa being an Account of their Medicinal and other Uses, Chemical Composition, Pharmacological Effects and Toxicology in Man and Animal.
Wen, Y., Chitwood, D.J., Vinyard, B.T., Bai, W., Meyer, S.L.F., 2017. Suppression of *Meloidogyne incognita* by extracts and powdered fruit of *Gleditsia sinensis* (Chinese honeylocust). Nematropica 47.
WHO. 2019. *International Programme on Chemical Safety* [Online]. Available: www.who.int [Accessed 20 April 2019].
Wiles, I.D., 2011. Chromatographic and Electrophoretic Separations Combined with Mass Spectrometry for Metabonomics. In: Lindon, JC, Nicholson, JK & Holmes, E (eds.) The Handbook of Metabonomics and Metabolomics. Elsevier B.V.
Williams, A.R., Soelberg, J., Jäger, A.K., 2016. Anthelmintic properties of traditional African and Caribbean medicinal plants: identification of extracts with potent activity against *Ascaris suum in vitro*. Parasite 23.

Williams, V.L., Raimondo, D., Crouch, N.R., Cunningham, A.B., Scott-Shaw, C.R., Lötter, M., Ngwenya, A.M. 2008. *Curtisia dentata (Burm.f.) C.A.Sm. National Assessment: Red List of South African Plants version 2020.1* [Online]. [Accessed 16 April 2019].

Williamson, V.M., Gleason, C.A., 2003. Plant–nematode interactions. Current Opinion in Plant Biology 6, 327-333.

Wink, M., van Wyk, B.-E., 2008. Mind-altering and poisonous plants of the world. Briza, Pretoria, South Africa.

Wintola, O., Afolayan, A., 2010. Ethnobotanical survey of plants used for the treatment of constipation within Nkonkobe Municipality of South Africa. African Journal of Biotechnology 9, 7767-7770.

Wu, H., Li, J., Fronczek, F.R., Ferreira, D., Burandt Jr, C.L., Setola, V., Roth, B.L., Zjawiony, J.K., 2013. Labdane diterpenoids from *Leonotis leonurus*. Phytochemistry 91, 229-235.

Wuyts, N., Swennen, R., De Waele, D., 2006. Effects of plant phenylpropanoid pathway products and selected terpenoids and alkaloids on the behaviour of the plant-parasitic nematodes *Radopholus similis*, *Pratylenchus penetrans* and *Meloidogyne incognita*. Nematology 8, 89-101.

Wyss, U., Grundler, F., 1992. Feeding behavior of sedentary plant parasitic nematodes. Netherlands Journal of Plant Pathology 98, 165-173.

Yakoob, A.T., Tajuddin, N.B., Hussain, M.I.M., Mathew, S., Govindaraju, A., Qadri, I., 2016. Antioxidant and hypoglycemic activities of *Clausena anisata* (Willd.) Hook F. ex Benth. root mediated synthesized silver nanoparticles. Pharmacognosy Journal 8.

Youssef, M., Eissa, M., 2014. Biofertilizers and their role in management of plant parasitic nematodes. A review. Journal of Biotechnology and Pharmaceutical Research 5, 1-6.

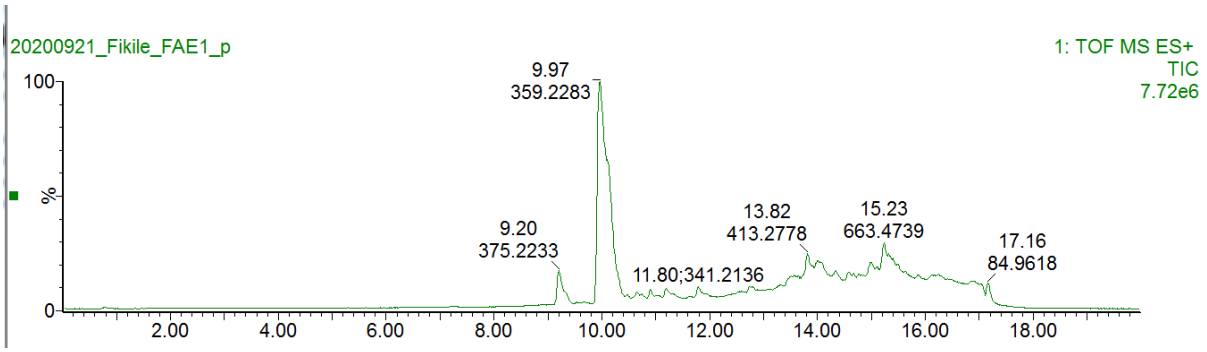
Youssef, M.M.A., Lashein, A.M.S., 2013. Effect of cabbage (*brassica oleracea*) leaf residue as a biofumigant, on root knot nematode, *Meloidogyne incognita* infecting tomato. Journal of Plant Protection Research 53, 271-274.

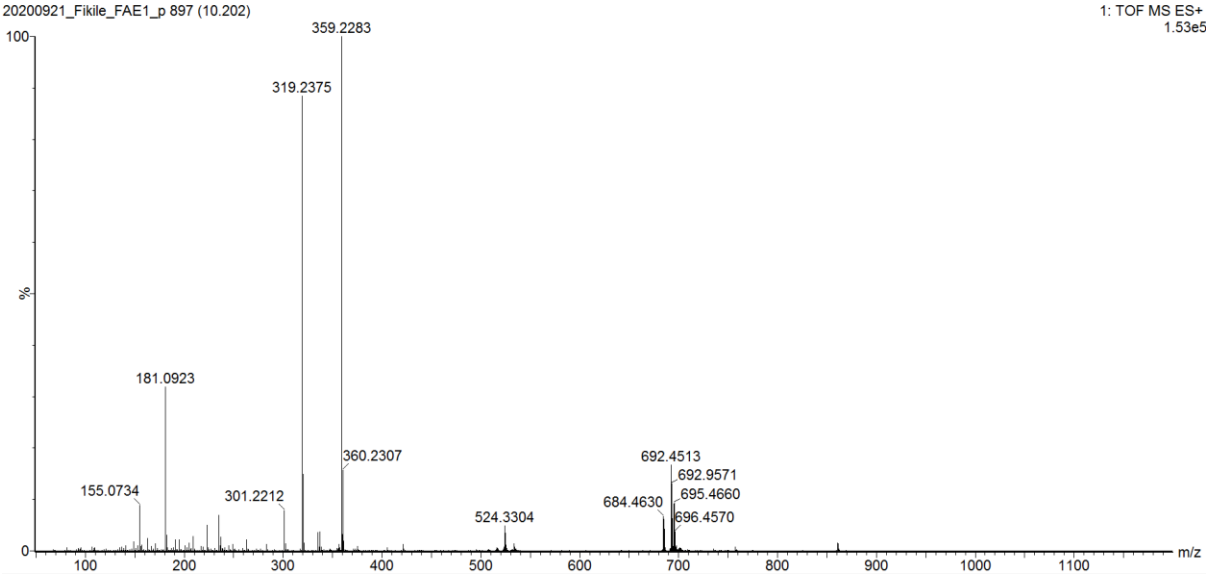
Zhang, L., Yan, C., Guo, Q., Zhang, J., Ruiz-Menjivar, J., 2018. The impact of agricultural chemical inputs on environment: global evidence from informetrics analysis and visualization. International Journal of low-Carbon technologies 13, 338-352.

Zijlstra, C., 2000. Identification of *Meloidogyne chitwoodi*, *M*. *fallax* and *M*. *hapla* based on SCAR-PCR: a powerful way of enabling reliable identification of populations or individuals that share common traits. European Journal of Plant Pathology 106, 283-290.

Appendix

Appendix 5.1: Compound Leoleorin C Mass Analysis





a

HRTOFMS spectrum (ESI^+)

Compound Leoleorin C Mass Analysis

Single Mass Analysis
Tolerance = 3.0 mDa / DBE: min = -1.5, max = 50.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 3
Monoisotopic Mass, Even Electron Ions
141 formula(e) evaluated with 2 results within limits (up to 10 closest results for each mass)
Elements Used:

Mass	Calc. Mass	mDa	PPM	DBE	Formula	i-FIT	i-FIT Norm	Fit Conf %	Frag. Mass	Fragment details	C	H	O	Na
359.2199	359.2198	0.1	0.3	4.5	C20 H32 O4 Na	23...	0.002	99.81			20	32	4	1
	359.2222	-2.3	-6.4	7.5	C22 H31 O4	23...	6.244	0.19			22	31	4	

Appendix 5.2: Elemental composition report for the compounds in the crude extracts

Leoleorin F

Single Mass Analysis
Tolerance = 5.0 mDa / DBE: min = -1.5, max = 50.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 3
Monoisotopic Mass, Even Electron Ions
1443 formula(e) evaluated with 2 results within limits (up to 10 closest results for each mass)
Elements Used:

Mass	Calc. Mass	mDa	PPM	DBE	Formula	i-FIT	i-FIT Norm	Fit Conf %	Frag. Mass	Fragment details	C	H	N	O	Na
338.3409	338.3399	1.0	3.0	-1.5	C20 H45 N O Na	66.8	0.057	94.45			20	45	1	1	1
	338.3423	-1.4	-4.1	1.5	C22 H44 N O	69.6	2.892	5.55			22	44	1	1	

Compund x

Single Mass Analysis
Tolerance = 5.0 mDa / DBE: min = -1.5, max = 50.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 3
Monoisotopic Mass, Even Electron Ions
126 formula(e) evaluated with 2 results within limits (up to 10 closest results for each mass)
Elements Used:

Mass	Calc. Mass	mDa	PPM	DBE	Formula	i-FIT	i-FIT Norm	Fit Conf %	Frag. Mass	Fragment details	C	H	O	Na
349.2010	349.2015	-0.5	-1.4	6.5	C20 H29 O5	65.4	2.188	11.22	141.0813	C4 H13 O5 : 5.0 (35.4)	20	29	5	
	349.1991	1.9	5.4	3.5	C18 H30 O5 Na	63.3	0.119	88.78	141.0813	C4 H13 O5 : 5.0 (35.4)	18	30	5	1

Leoleorin H

Single Mass Analysis
Tolerance = 5.0 mDa / DBE: min = -1.5, max = 50.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 3
Monoisotopic Mass, Even Electron Ions
275 formula(e) evaluated with 3 results within limits (up to 10 closest results for each mass)
Elements Used:

Mass	Calc. Mass	mDa	PPM	DBE	Formula	i-FIT	i-FIT Norm	Fit Conf %	C	H	O	Na	K
379.2471	379.2460	1.1	2.9	2.5	C20 H36 O5 Na	11...	0.031	96.92	20	36	5	1	
	379.2484	-1.3	-3.4	5.5	C22 H35 O5	11...	3.479	3.08	22	35	5		
	379.2426	4.5	11.9	14.5	C29 H31	12...	10.813	0.00	29	31			

Leoleorin B

Single Mass Analysis

Tolerance = 5.0 mDa / DBE: min = -1.5, max = 50.0

Element prediction: Off

Number of isotope peaks used for i-FIT = 3

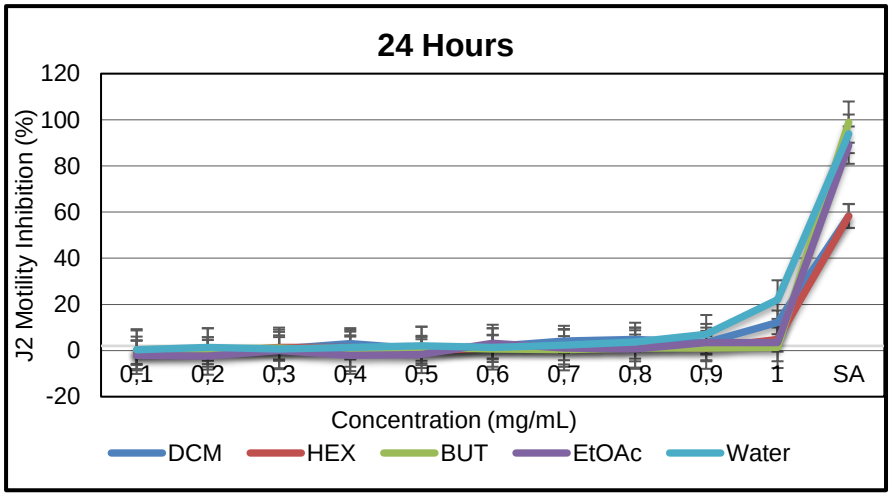
Monoisotopic Mass, Even Electron Ions

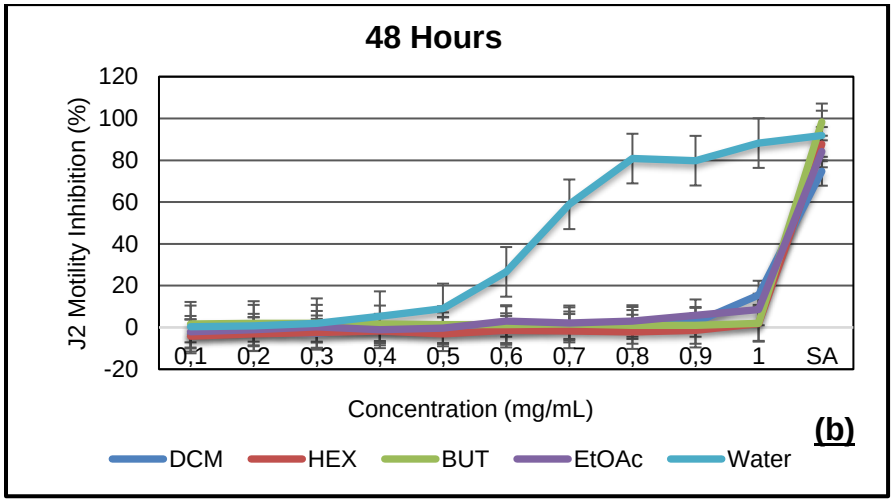
104 formula(e) evaluated with 2 results within limits (up to 10 closest results for each mass)

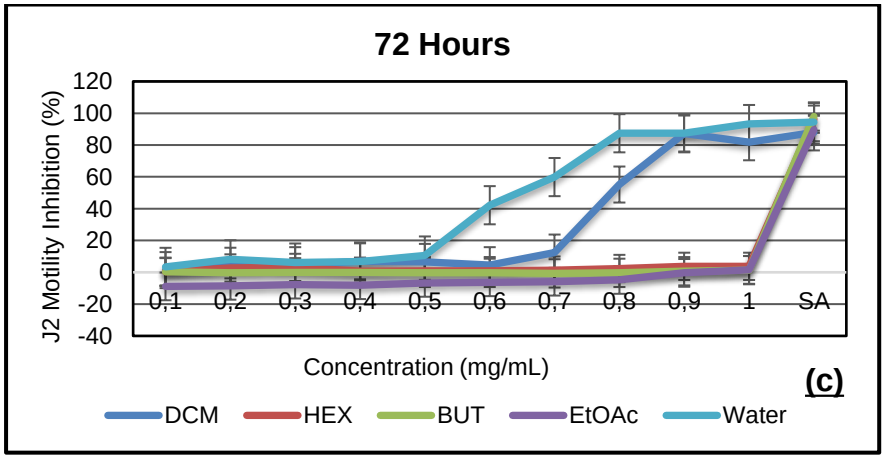
Elements Used:

Mass	Calc. Mass	mDa	PPM	DBE	Formula	i-FIT	i-FIT Norm	Fit Conf %	Frag. Mass	Fragment details	C	H	O	Na
299.2002	299.2011	-0.9	-3.0	7.5	C20 H27 O2	98.7	1.516	21.95			20	27	2	
	299.1987	1.5	5.0	4.5	C18 H28 O2 Na	97.4	0.248	78.05			18	28	2	1

Appendix 5.3: Effect of *Leonotis leonurus* fractions against motility of *M. incognita* J2 exposed at 24, 48 and 72 hours

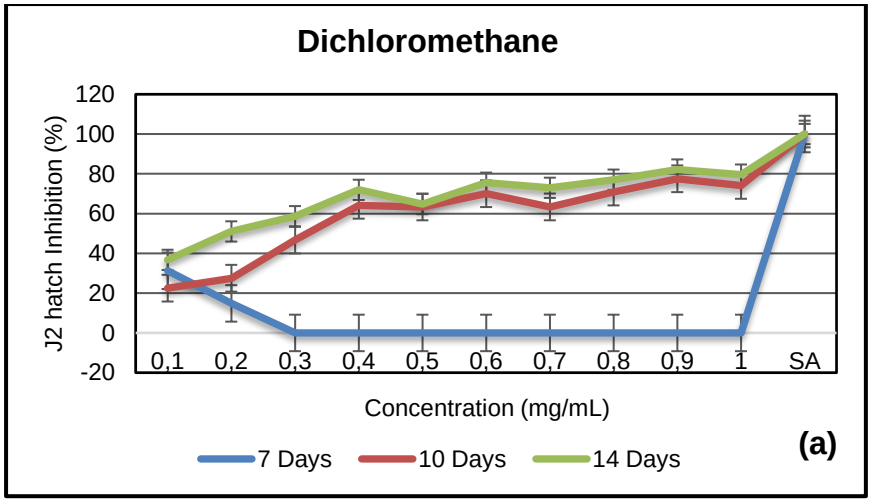


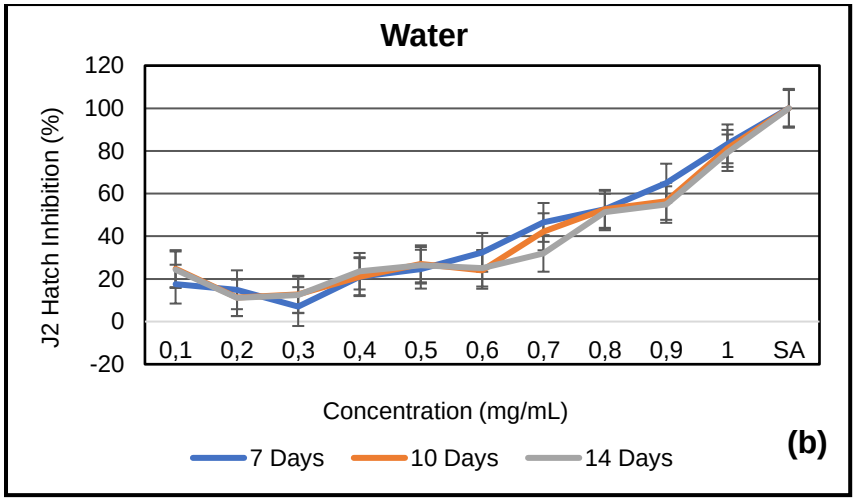


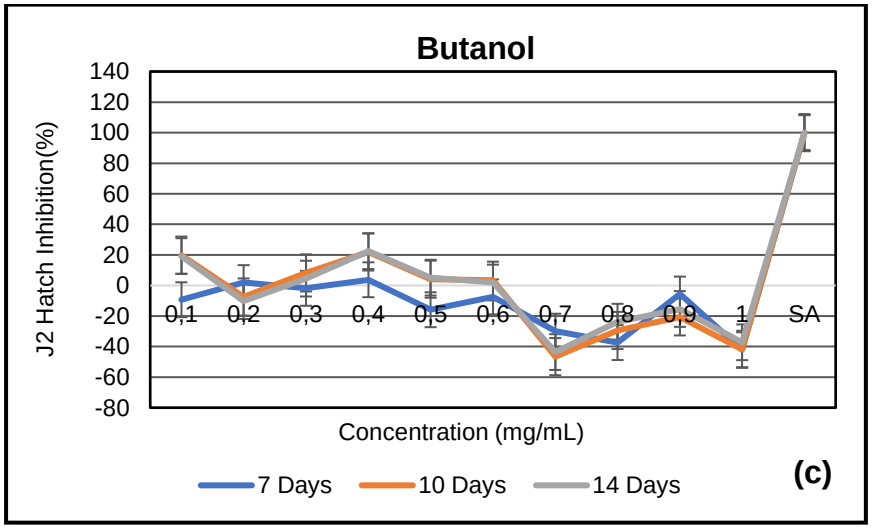


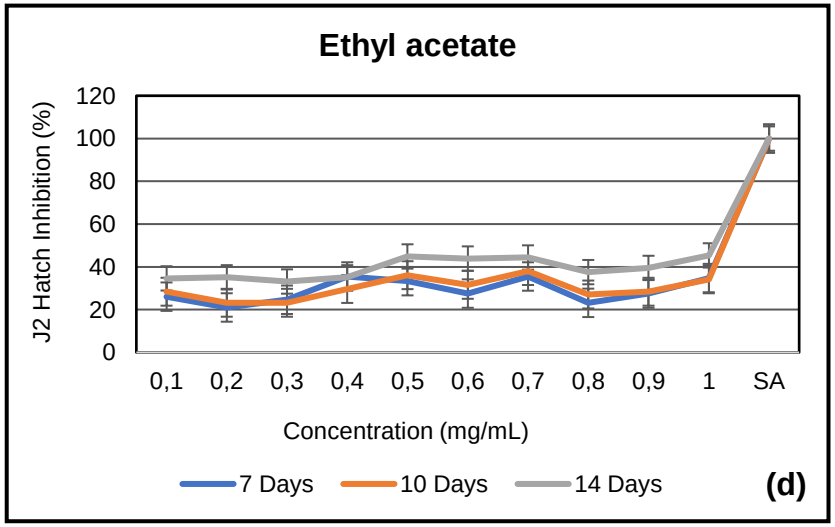
DCM-dichloromethane; HEX-*n*-hexane, BUT-*n*-Butanol, EtOAc-Ethyl acetate

Figure 5.8: Effect of *Leonotis leonurus* fractions against motility of *M. incognita* J2 exposed at 24, 48 and 72 hours.


Dichloromethane
J2 hatch Inhibition (%)
120
100
80
60
40
20
0
-20
0,1 0,2 0,3 0,4 0,5 0,6 0,7 0,8 0,9 1 SA
Concentration (mg/mL)
7 Days
10 Days
14 Days
(a)


Water
J2 Hatch Inhibition (%)
120
100
80
60
40
20
0
-20
0,1 0,2 0,3 0,4 0,5 0,6 0,7 0,8 0,9 1 SA
Concentration (mg/mL)
7 Days
10 Days
14 Days
(b)


Butanol
J2 Hatch Inhibition(%)
140
120
100
80
60
40
20
0
-20
-40
-60
-80
0,1 0,2 0,3 0,4 0,5 0,6 0,7 0,8 0,9 1 SA
Concentration (mg/mL)
7 Days
10 Days
14 Days
(c)


Ethyl acetate
J2 Hatch Inhibition (%)
120
100
80
60
40
20
0
0,1 0,2 0,3 0,4 0,5 0,6 0,7 0,8 0,9 1 SA
Concentration (mg/mL)
7 Days
10 Days
14 Days
(d)

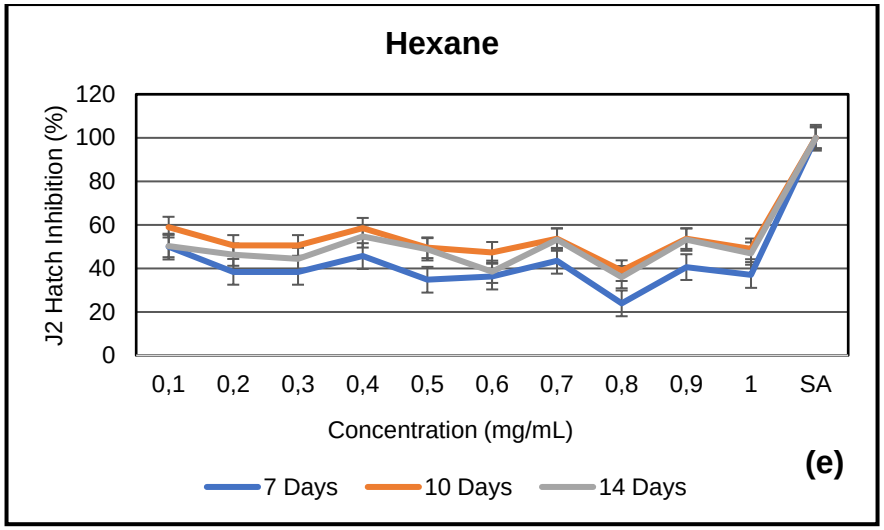
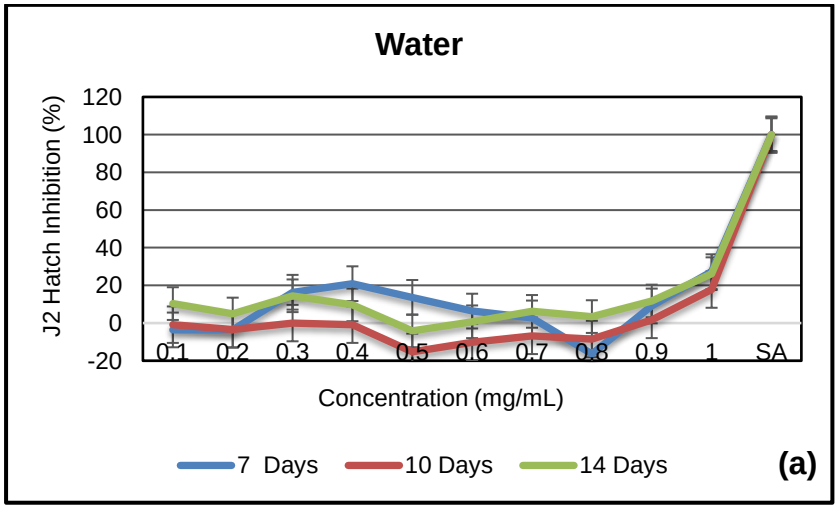
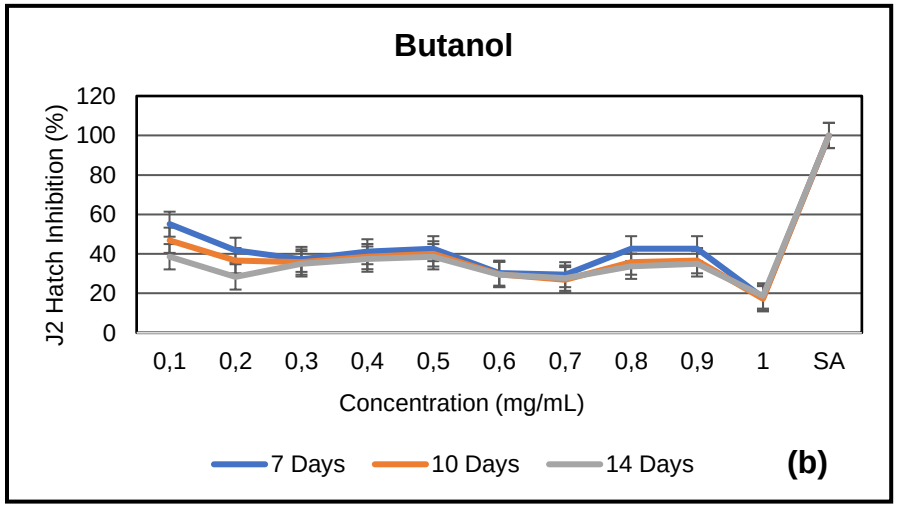
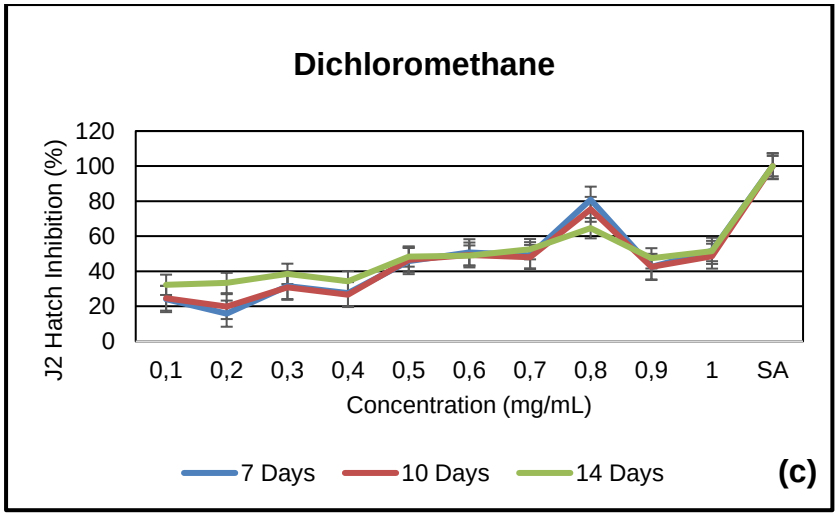
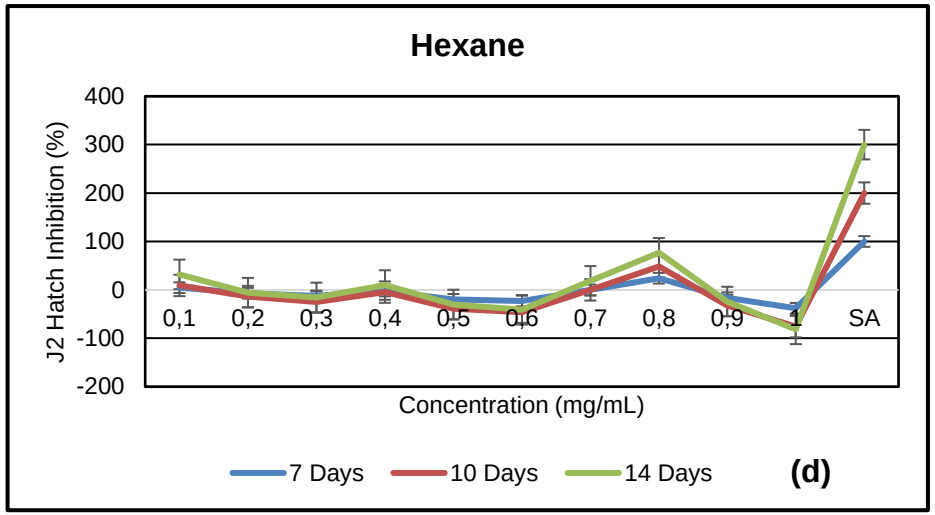


Figure 5.9: Effect of *Leonotis leonurus* fractions against J2 hatch inhibition of *M. incognita* exposed at 7, 10 and 14 days









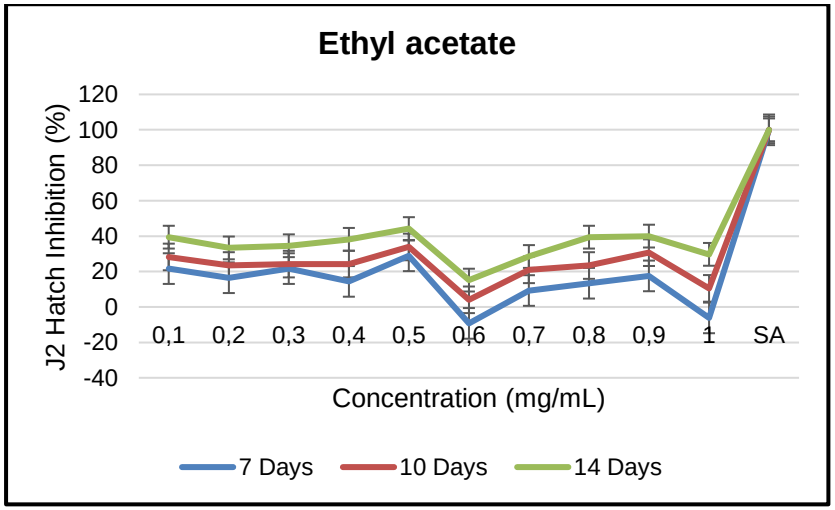


Figure 5.10: Effect of *Clausena anisata* fractions against J2 hatch inhibition of *M. incognita* exposed at 7, 10 and 14 days

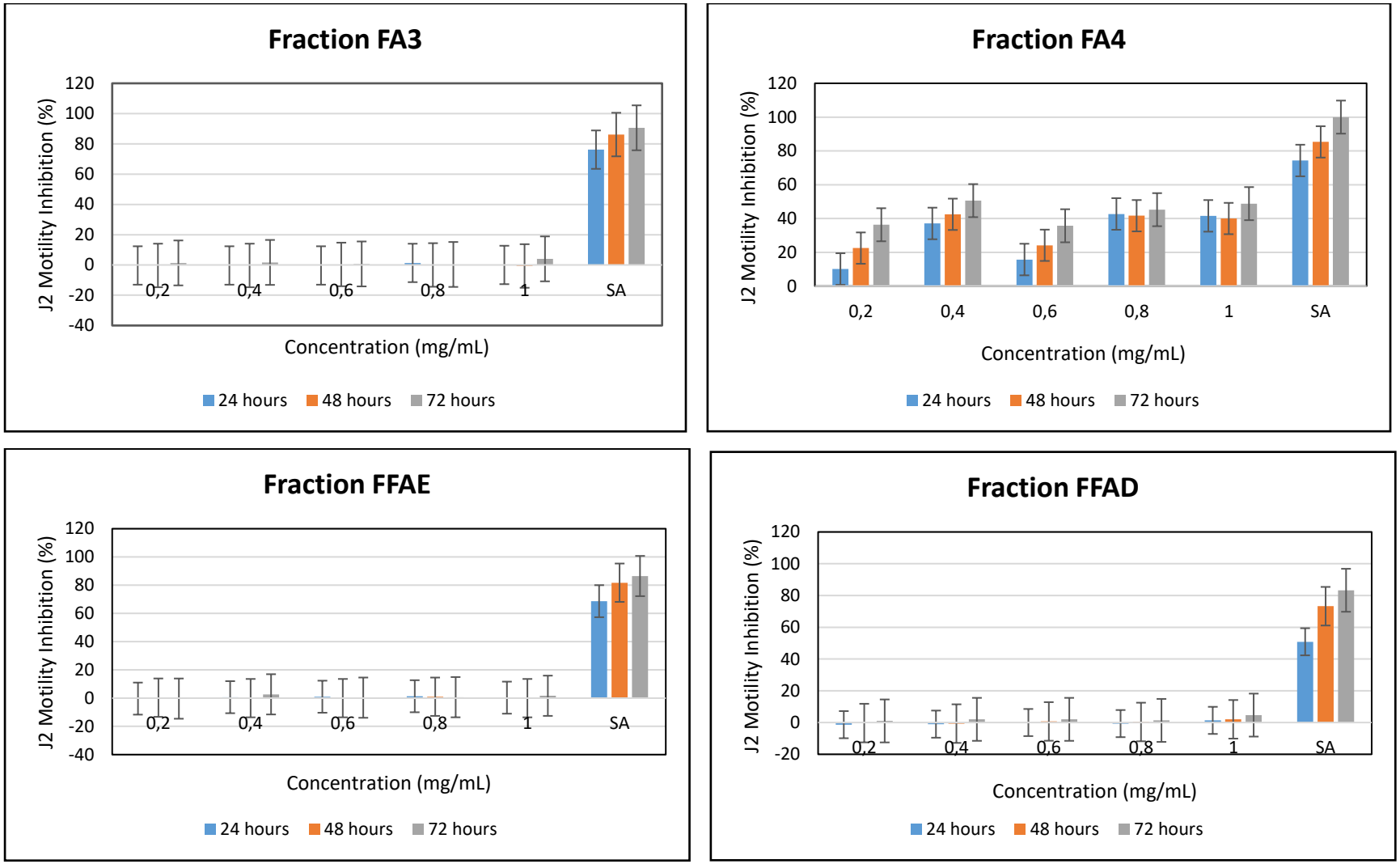


Figure 5.11: Effect of *L. leonurus* subfractions against motility of *M. incognita* exposed at 24, 48 and 72 hours.

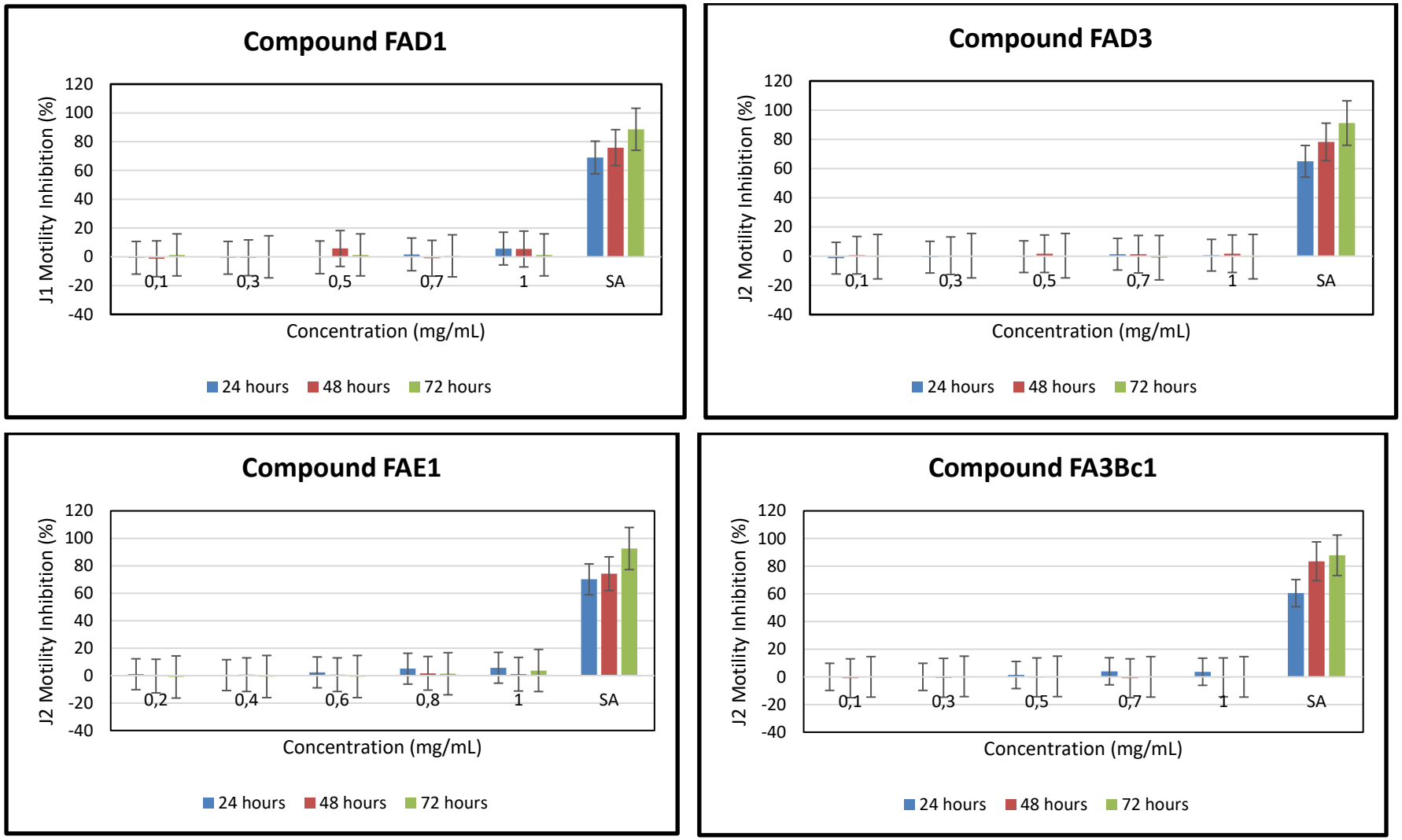


Figure 5.12: Effect of the isolated compounds against motility of *M. incognita* exposed at 24, 48 and 72 hours

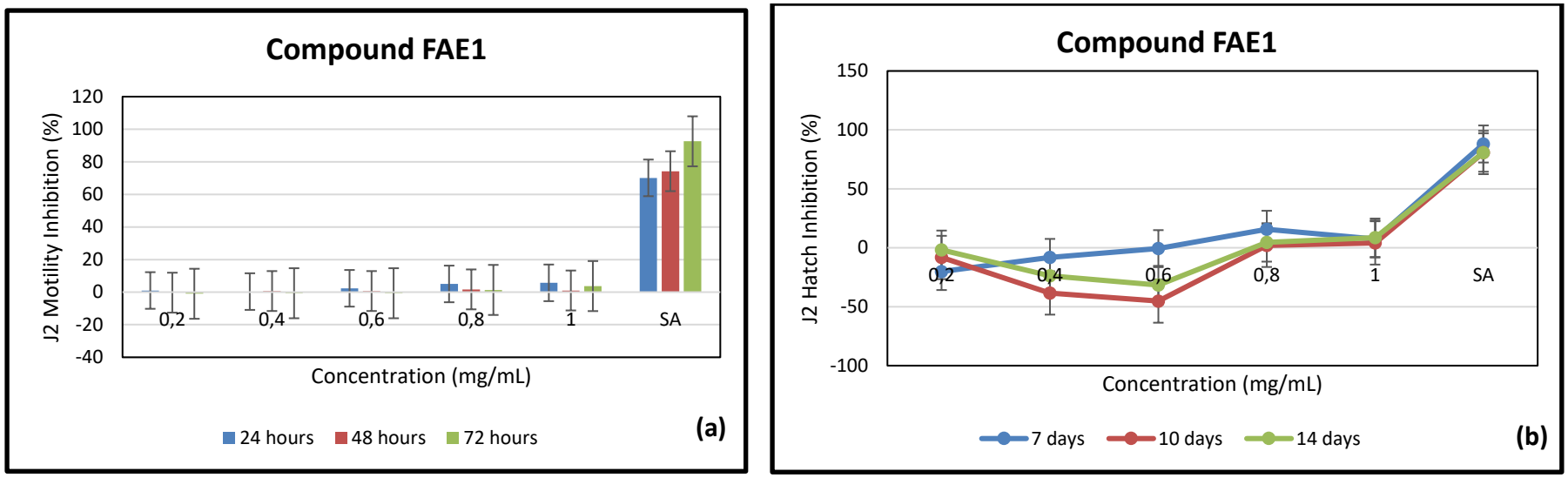


Figure 5.13: Effect of compound FAE1 against motility (a) and J2 hatch inhibition (b) of *M. incognita*

Appendix 6.

Phytotoxicity *in vitro* experiments

Appendix 6.1.1: Completely Randomized AOV for radicle of tomato seedling in Trials

Source	DF	SS	MS	F	P
Trial	1	8,26	8,26298	5,39	0,0205
Error	718	1099,89	1,53187		
Total	719	1108,15			

Appendix 6.1.2: Completely Randomized AOV for plumule of tomato seedling in Trials

Source	DF	SS	MS	F	P
Trial	1	2333,38	2333,38	394,26	0
Error	718	4249,35	5,92		
Total	719	6582,73			

Appendix 6.1.3: Completely Randomized AOV for height of tomato seedling in Trials

Source	DF	SS	MS	F	P
Trial	1	11,02	11,0182	5,19	0,023
Error	718	1524,19	2,1228		
Total	719	1535,21			

Appendix 6.1.4: Completely Randomized AOV for dry weight of tomato seedling in Trials

Source	DF	SS	MS	F	P
Trial	1	1,126	1,12646	1,69	0,1939
Error	718	478,334	0,6662		
Total	719	479,461			

Appendix 6.1.5: Factorial ANOVA for plumule of tomato seedling in experiment 1

Source	DF	SS	MS	F	P
Seedlings	9	1,17307	0,13034		
Concentration	3	0,38116	0,12705	14,07	0
Plant name	2	0,15974	0,07987	8,85	0,0002
Concentration*Plant name	6	0,29287	0,04881	5,41	0
Error	339	3,06044	0,00903		
Total	359	5,06727			

Appendix 6.1.6: Factorial ANOVA for radicle of tomato seedling in experiment 1

Source	DF	SS	MS	F	P
Seedlings	9	3,0453	0,33837		
Concentration	3	6,1677	2,05591	98,02	0
Plant name	2	1,0709	0,53546	25,53	0
Concentration*Plant name	6	0,7457	0,12429	5,93	0
Error	339	7,1102	0,02097		
Total	359	18,1399			

Appendix 6.1.7: Factorial ANOVA for the height of tomato seedling in experiment 1

Source	DF	SS	MS	F	P
Seedlings	9	4,6376	0,51528		
Concentration	3	5,8909	1,96362	65,86	0
Plant name	2	0,9665	0,48325	16,21	0
Concentration*Plant name	6	0,9008	0,15013	5,04	0,0001
Error	339	10,1078	0,02982		
Total	359	22,5035			

Appendix 6.1.8: Factorial ANOVA for the weight of tomato seedling in experiment 1

Source	DF	SS	MS	F	P
Seedlings	9	1,8688	0,20764		
Concentration	3	1,8918	0,6306	31,11	0
Plant name	2	0,1092	0,05458	2,69	0,0692
Concentration*Plant name	6	0,7264	0,12107	5,97	0
Error	339	6,8721	0,02027		
Total	359	11,4683			

Appendix 6.1.9: Factorial ANOVA for plumule of tomato seedling in experiment 2

Source	DF	SS	MS	F	P
Seedlings	8	6,8497	0,85621		
Concentratiom	3	0,54311	0,18104	7,33	0,0001
Plant	2	0,76487	0,38244	15,49	0
Concentration*Plant	6	1,3638	0,2273	9,21	0
Error	340	8,39428	0,02469		
Total	359				

Appendix 6.1.10: Factorial ANOVA for radicle of tomato seedling in experiment 2

Source	DF	SS	MS	F	P
Seedlings	8	10,7379	1,34224		
Concentration	3	10,5325	3,51084	69,59	0
Plant	2	4,4888	2,24439	44,49	0
Concentration*Plant	6	3,1075	0,51792	10,27	0
Error	340	17,1539	0,05045		
Total	359				

Appendix 6.1.11: Factorial ANOVA for height of tomato seedling in experiment 2

Source	DF	SS	MS	F	P
Seedlings	8	8,69052	1,08631		
Concentration	3	0,68509	0,22836	8,3	0
Plant	2	0,96875	0,48437	17,6	0
Concentration*Plant	6	1,34776	0,22463	8,16	0
Error	340	9,35784	0,02752		
Total	359				

Appendix 6.1.12: Factorial ANOVA for dried weight of tomato seedling in experiment 2

Source	DF	SS	MS	F	P
Seedlings	8	3,28826	0,41103		
Concentration	3	0,49561	0,1652	9,37	0
Plant	2	0,13427	0,06714	3,81	0,0232
Concentration*Plant	6	0,33806	0,05634	3,19	0,0046
Error	340	5,9975	0,01764		
Total	359				

Glasshouse experiments

Trial 1

Appendix 6.2.1: Analysis of variance (ANOVA) of split plot of dosage and treatment on gall index of tomato plants

Source	DF	SS	MS	F	P
Replication	3	0,09248	0,03083		
Treatment (TMT)	4	0,57478	0,1437	3,85	0,0094
Dosage	2	1,02319	0,51159	13,71	0
TMT*Dosage	8	0,26313	0,03289	0,88	0,5398
Error	42	1,56687	0,03731		
Total	59	3,52045			

Appendix 6.2.2: Analysis of variance (ANOVA) of split plot of dosage and treatment on root mass of tomato plants

Source	DF	SS	MS	F	P
Replication	3	1137	379,01		
Treatment (TMT)	4	4719,9	1179,98	2,16	0,0904
Dosage	2	15587,5	7793,74	14,25	0
TMT*Dosage	8	6294,8	786,85	1,44	0,2091
Error	42	22967,2	546,84		
Total	59	50706,4			

Appendix 6.2.3: Analysis of variance (ANOVA) of split plot of dosage and treatment on stem height of tomato plants

Source	DF	SS	MS	F	P
Replication	3	0,14533	0,04844		
Treatment (TMT)	4	0,22924	0,05731	0,76	0,5542
Dosage	2	0,2297	0,11485	1,53	0,2278
TMT*Dosage	8	0,65311	0,08164	1,09	0,3894
Error	42	3,14757	0,07494		
Total	59	4,40494			

Appendix 6.2.4: Analysis of variance (ANOVA) of split plot of dosage and treatment on wet shoot mass of tomato plants

Source	DF	SS	MS	F	P
Replication	3	0,22919	0,0764		
Treatment (TMT)	4	0,25477	0,06369	0,61	0,6584
Dosage	2	0,0853	0,04265	0,41	0,6677
TMT*Dosage	8	0,89692	0,11212	1,07	0,4008
Error	42	4,39261	0,10459		
Total	59	5,85881			

Appendix 6.2.5: Analysis of variance (ANOVA) of split plot of dosage and treatment on dry shoot mass of tomato plants

Source	DF	SS	MS	F	P
Replication	3	0,07353	0,02451		
Treatment (TMT)	4	0,10679	0,0267	0,51	0,7321
Dosage	2	0,12294	0,06147	1,16	0,3224
TMT*Dosage	8	0,44387	0,05548	1,05	0,4156
Error	42	2,21975	0,05285		
Total	59	2,96688			

Appendix 6.2.6: Analysis of variance (ANOVA) of split plot of dosage and treatment on flowers of tomato plants

Source	DF	SS	MS	F	P
Replication	3	68,6	22,867		
Treatment (TMT)	4	347,5	86,875	1,29	0,2895
Dosage	2	185,83	92,917	1,38	0,2629
TMT*Dosage	8	981	122,625	1,82	0,1002
Error	42	2829,4	67,367		
Total	59	4412,33			

Appendix 6.2.7: Analysis of variance (ANOVA) of split plot of dosage and treatment on fruit number of tomato plants

Source	DF	SS	MS	F	P
Replication	3	0,06455	0,02152		
Treatment (TMT)	4	0,35968	0,08992	2,29	0,076
Dosage	2	0,06469	0,03234	0,82	0,4464
TMT*Dosage	8	0,21007	0,02626	0,67	0,7167
Error	42	1,65193	0,03933		
Total	59	2,35092			

Appendix 6.2.8: Analysis of variance (ANOVA) of split plot of dosage and treatment on fruit mass of tomato plants

Source	DF	SS	MS	F	P
Replication	3	34916	11638,5		
Treatment (TMT)	4	19032	4758	0,54	0,7081
Dosage	2	7244	3621,8	0,41	0,6662
TMT*Dosage	8	30183	3772,9	0,43	0,8981
Error	42	370934	8831,8		
Total	59	462308			

Appendix 6.2.9: Analysis of variance (ANOVA) of split plot of dosage and treatment on stem diameter of tomato plants

Source	DF	SS	MS	F	P
Replication	3	0,05869	0,01956		
Treatment (TMT)	4	0,09188	0,02297	1,24	0,3099
Dosage	2	0,04456	0,02228	1,2	0,3113
TMT*Dosage	8	0,08561	0,0107	0,58	0,7913
Error	42	0,77981	0,01857		
Total	59	1,06055			

Appendix 6.2.10: Analysis of variance (ANOVA) of split plot of dosage and treatment on eggs

Source	DF	SS	MS	F	P
Replication	3	14,057	4,6858		
Treatment (TMT)	4	22,815	5,7037	4,6	0,0036
Dosage	2	28,407	14,2033	11,46	0,0001
TMT*Dosage	8	7,581	0,9476	0,76	0,6355
Error	42	52,067	1,2397		
Total	59	124,926			

Appendix 6.2.11: Analysis of variance (ANOVA) of split plot of dosage and treatment on juveniles

Source	DF	SS	MS	F	P
Replication	3	9,749	3,24972		
Treatment (TMT)	4	16,123	4,03071	3,27	0,02
Dosage	2	18,373	9,18648	7,46	0,0017
TMT*Dosage	8	8,899	1,11234	0,9	0,5225
Error	42	51,703	1,23102		
Total	59	104,847			

Trial 2

Appendix 6.2.12: Analysis of variance (ANOVA) of split plot of dosage and treatment on gall index of tomato plants

Source	DF	SS	MS	F	P
Replication	3	0,5333	0,17778		
Treatment (TMT)	4	34,2667	8,56667	65,82	0
Dosage	2	2,1333	1,06667	8,2	0,001
TMT*Dosage	8	4,5333	0,56667	4,35	0,0007
Error	42	5,4667	0,13016		
Total	59	46,9333			

Appendix 6.2.13: Analysis of variance (ANOVA) of split plot of dosage and treatment on fruit number of tomato plants

Source	DF	SS	MS	F	P
Replication	3	6,1833	2,06111		
Treatment (TMT)	4	4,1	1,025	2	0,1126
Dosage	2	1,2333	0,61667	1,2	0,311
TMT*Dosage	8	3,1	0,3875	0,75	0,6437
Error	42	21,5667	0,51349		
Total	59	36,1833			

Appendix 6.2.14: Analysis of variance (ANOVA) of split plot of dosage and treatment on fruit mass of tomato plants

Source	DF	SS	MS	F	P
Replication	3	44,597	14,8658		
Treatment (TMT)	4	58,498	14,6246	2,39	0,0656
Dosage	2	27,426	13,7132	2,25	0,1185
TMT*Dosage	8	45,864	5,733	0,94	0,4956
Error	42	256,528	6,1078		
Total	59	432,913			

Appendix 6.2.15: Analysis of variance (ANOVA) of split plot of dosage and treatment on stem diameter of tomato plants

Source	DF	SS	MS	F	P
Replication	3	0,04271	0,01424		
Treatment (TMT)	4	0,11342	0,02836	2,57	0,0514
Dosage	2	0,00066	0,00033	0,03	0,9704
TMT*Dosage	8	0,09059	0,01132	1,03	0,4308
Error	42	0,46271	0,01102		
Total	59	0,7101			

Appendix 6.2.16: Analysis of variance (ANOVA) of split plot of dosage and treatment on stem height of tomato plants

Source	DF	SS	MS	F	P
Replication	3	6390,3	2130,1		
Treatment (TMT)	4	1591,3	397,83	1,29	0,2879
Dosage	2	2830	1414,98	4,6	0,0156
TMT*Dosage	8	3462	432,74	1,41	0,2218
Error	42	12915,2	307,51		
Total	59	27188,8			

Appendix 6.2.17: Analysis of variance (ANOVA) of split plot of dosage and treatment on wet shoot of tomato plants

Source	DF	SS	MS	F	P
Replication	3	7343,2	2447,73		
Treatment (TMT)	4	32866,4	8216,6	7,27	0,0002
Dosage	2	4299,1	2149,53	1,9	0,1619
TMT*Dosage	8	6103,1	762,89	0,67	0,7105
Error	42	47469,5	1130,23		
Total	59	98081,3			

Appendix 6.2.18: Analysis of variance (ANOVA) of split plot of dosage and treatment on dry shoot of tomato plants

Source	DF	SS	MS	F	P
Replication	3	31,27	10,424		
Treatment (TMT)	4	1009,93	252,482	7,72	0,0001
Dosage	2	42,45	21,225	0,65	0,5278
TMT*Dosage	8	211,26	26,407	0,81	0,6
Error	42	1373,97	32,714		
Total	59	2668,87			

Appendix 6.2.19: Analysis of variance (ANOVA) of split plot of dosage and treatment on number of flowers of tomato plants

Source	DF	SS	MS	F	P
Replication	3	176,85	58,95		
Treatment (TMT)	4	387,93	96,983	1,78	0,151
Dosage	2	249,43	124,717	2,29	0,1139
TMT*Dosage	8	337,07	42,133	0,77	0,6282
Error	42	2288,9	54,498		
Total	59	3440,18			

Appendix 6.2.20: Analysis of variance (ANOVA) of split plot of dosage and treatment on roots of tomato plants

Source	DF	SS	MS	F	P
Replication	3	52,84	17,615		
Treatment (TMT)	4	1778,35	444,589	5,21	0,0017
Dosage	2	315,05	157,523	1,85	0,1704
TMT*Dosage	8	997,92	124,74	1,46	0,2001
Error	42	3582,74	85,303		
Total	59	6726,9			

Appendix 6.2.21: Analysis of variance (ANOVA) of split plot of dosage and treatment on eggs

Source	DF	SS	MS	F	P
Replication	3	0,2046	0,06821		
Treatment (TMT)	4	22,1708	5,5427	28,96	0
Dosage	2	0,3142	0,15708	0,82	0,4471
TMT*Dosage	8	2,2709	0,28386	1,48	0,1925
Error	42	8,0398	0,19142		
Total	59	33,0002			

Appendix 6.2.22: Analysis of variance (ANOVA) of split plot of dosage and treatment on juveniles

Source	DF	SS	MS	F	P
Replication	3	1,8099	0,6033		
Treatment (TMT)	4	66,4074	16,6018	38,8	0
Dosage	2	0,6905	0,3453	0,81	0,453
TMT*Dosage	8	8,1942	1,0243	2,39	0,0317
Error	42	17,9701	0,4279		
Total	59	95,0721			