Ameliorative effects of botanicals and rhizobacteria on the growth of *Pelargonium sidoides* and *Solanum lycopersicum* infested with *Meloidogyne incognita*

By

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Submitted in fulfilment of the requirements for the degree of Doctor of Philosophy

Research Centre for Plant Growth and Development

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College of Agriculture, Engineering and Science

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Regular consultation took place w d the investigation.

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Publications from this Thesis

1. **N.T. Sithole, M.G. Kulkarni, J.F. Finnie, J. Van Staden. 2021.** Potential nematicidal properties of plant extracts against *Meloidogyne incognita.* South African Journal of Botany, 139: 409-417.
2. **N.T. Sithole, S. Gupta, M.G. Kulkarni, J.F. Finnie, J. Van Staden. 2021.** Effects of botanicals on growth and phytochemistry of the nematode-infected *Pelargonium sidoides* and GC-MS profiling of *Cucurbita maxima* seeds (Under review; Journal of Plant Growth Regulation).
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Declaration 2 – Publications

DETAILS OF CONTRIBUTION TO PUBLICATIONS that form part and/or include research presented in this thesis (include publications in preparation, submitted, in press and published and give details of the contributions of each author to the experimental work and writing of each publication).

Publication 1 Contributions: Experimental work and draft manuscript were done by NTS. MGK assisted proof-reading the manuscript. JFF and JVS supervised the whole study and edited the manuscript before submission.

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Publication 3 Contributions: Experimental work and draft manuscript were done by NTS. SG and MGK assisted with experimental work and proof-read the manuscript. JFF and JVS supervised the whole study and edited the manuscript before submission.

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Conference Contributions from this Thesis

1. **N.T. Sithole, S. Gupta, M.G. Kulkarni, J.F. Finnie, J. Van Staden**. Bio-nematicidal properties of eight medicinal plant extracts for controlling root-knot nematodes (*Meloidogyne incognita*) infestation. 45th Annual SAAB, AMA & SASSB Joint Congress, Johannesburg, South Africa, 08-11 January 2019.
2. **N.T. Sithole, S. Gupta, M.G. Kulkarni, J.F. Finnie, J. Van Staden**. Influence of grounded and drenching applications of medicinal plant biomass on plant physiology and morphology of nematode-infested *Pelargonium sidoides*. 46thAnnual SAAB, AMA & SASSB Joint Congress, Qwa-Qwa, South Africa, 07-10 January 2020.
3. **N.T. Sithole, S. Gupta, M.G. Kulkarni, J.F. Finnie, J. Van Staden.** Synergistic interaction of ground biomass of medicinal plants and rhizobacteria for the management of *Meloidogyne Incognita* on *Solanum Lycopersicum* L. 7th International Congress of Nematology. Conference center of Antibes Juan-Les-Pins, France, 1-6 May 2022.

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Dedication

To my late beloved sister Mrs. Thandi Busisiwe Sithole-Malumane whom I wish was here to witness this day. Sis, you selflessly sacrificed so much in life for our future, you will forever be in our thoughts.

"Nkundlandla

Sen'wayo

Nkomo

Mbuqundaka"

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List of Abbreviations

AA/ABA	Abscisic acid
ACC	Aminocyclopropane-1-carboxylate
ANOVA	Analysis of variance
ATCC	American Type Culture Collection
BC	Biological control
BHT	Butylated hydroxytoluene
BSA	Bovine serum albumin
BS	Bacterial suspensions
CAO	Chlorophyllide a oxygenase
CCE	Cyanide chloride equivalent
CE	Catechin equivalent
CFU	Colony-forming unit
CH_4	Methane
DCM	Dichloromethane
DMSO	Dimethyl sulfoxide
DPPH	2,2-diphenyl-1-picrylhydrazyl
DW	Dry weight
DF	Dworkin and Foster
DDG	Density-dependent growth
EtOAc	Ethyl acetate
FW	Fresh weight
GAE	Gallic acid equivalents
GC-MS	Gas Chromatography-Mass Spectrometry
GHGs	Greenhouse gases
N_2O	Nitrogen oxide
HCN	Hydrogen cyanide
HCL	Hydrochloric acid
HDTMA	Hexadecyltrimethylammonium
IAA	Indole-3-acetic acid
IC_{50}	50% inhibition concentration
IPCC	International Panel of Climate Change
INT	p-Idonitro tetrazolium chloride
IPM	Integrated pest management
ISR	Induced systemic resistance
J1	First-stage juveniles
J2	Second-stage juveniles
J3	Third-stage juveniles
J4	Fourth-stage juveniles
KCl	Potassium chloride
KH_2O_2	Potassium hydrogen peroxide
LB	Luria Bertani
MH	Mueller-Hinton
MIC	Minimum inhibition concentration
MS	Mass spectrometer
NA	Nutrient agar
NaCl	Sodium chloride
Na_2HPO_4	Sodium hydrogen phosphate
NaOCl	Sodium hypochloride
Na_2CO_3	Sodium carbonate

$(NH_4)_2SO_4$	Ammonia sulfate
NH_3	Ammonia
NIST	National Institute of Standards and Technology
PBS	Phosphate–buffer saline
PE	Petroleum ether
PGPR	Plant growth-promoting rhizobacteria
Pf	Final population
Pi	Initial population
PPNs	Plant-parasitic nematodes
PSI	Phosphate solubilizing index
PVK	Pikovskaya's
QE	Quercetin equivalent
RF	Reproduction factor
RKNs	Root-knot nematodes
SMA	Skimmed milk agar
SE	Standard error
SPSS	Statistical Product and Service Solutions
TE	Trace elements
TTV	Total treatment variation
WHO	World Health Organization

Abstract

Among the most destructive pests affecting plant growth and yield worldwide are root-knot nematodes (*Meloidogyne* spp.). Currently, the shift from the use of chemical nematicides is progressing globally towards choices such as organic nematicides derived from plant substances and biological control agents. Secondary metabolites of plants and micro-organisms have been identified as bioactive compounds responsible for nematicidal activities in different plant and bacterium species, and are favored nematicidal modes because of their safe environmental records. Firstly, eight botanicals namely *Cucurbita maxima* Duchesne (Autumn squash)*, Prunus africana* (Hoof. f.) Kalkman (African cherry), *Pelargonium sidoides* DC. (South African geranium), *Croton sylvaticus* Hochst (Forest fever-berry)*, Solanum aculeastrum* Dunal (Poison apple), *Vernonia colorata* (Willd) Drake (Bitter leaf)*, Searsia lancea* (L.F.) F.A. Barkley (African sumac) and *Merwilla plumbea* (Lindl.) Speta (Blue squill) were selected based on their anthelmintic activity and then screened for their phytochemical and antioxidant activities. In addition, the mutagenic activity of these eight botanicals was evaluated against TA102 and TA1535 bacterial strains. Secondly, the *in vitro* antimicrobial properties of the eight botanicals were investigated, and subsequently the *in vitro* and *ex vitro* nematicidal capacities of the same plants were tested against root-knot nematode (*Meloidogyne incognita*) egg mass hatchability, second-stage juvenile (J2s) mobility and mortality activities. Thirdly, the study examined the *in vitro* plant growth-promoting attributes of *B. licheniformis* ATCC 12759 and *P. fluorescens* ATCC 13525 as well as their growth-promoting effects on tomato seedlings under greenhouse conditions. In addition, the study evaluated the impact of the eight finely ground plant materials at varying rates (0, 2, 6 and 10 g) against nematode infecting tomato seedlings. Furthermore, the study also evaluated the integration of *B. licheniformis* or *P. fluorescens* with the eight powdered

plant materials as a combination management strategy of the root-knot nematode *M. incognita* on tomato seedlings under greenhouse conditions. Fourthly, the study evaluated the nematicidal effects of plant materials on the physiological and biochemical attributes of *M. incognita* infected *P. sidoides* seedlings. Lastly, this study investigated the phytochemical bioactive compounds profile of *C. maxima* seeds using GC-MS as a potential source of phytonematicidal compounds.

The phytochemical analysis showed that the highest total flavonoids, condensed tannins and phenolic contents were found in *V. colorata* (88.71 mg QE/g), *S. lancea* (406.16 mg CCE/g) and *P. sidoides* (175.95 mg GAE/g), respectively. Plant extracts of methanol, water and petroleum ether exhibited an increase in DPPH free radical scavenging activity relative to concentration, with *P. sidoides*, *S. lancea*, *P. africana*, *S. aculeastrum* and *C. sylvaticus* significantly (p<0.01) yielded the highest antioxidant activity. On the other hand, the eight methanolic plant extracts demonstrated an increase in the number of His^+ revertants, ranging from 13.33 to 127.67, with increasing concentration against the TA102 bacterial strain, however, the number of the His^+ revertants were significantly less than those of the positive control (4NQO) (237.67). The results further revealed that all treatments with increasing concentrations suppressed egg mass hatching after 72 h. *Cucurbita maxima* methanolic crude extract with the least phytochemicals and antioxidant activity significantly reduced egg mass hatchability by 96% at 0.8 $mgmL^{-1}$ with 100% second-stage juvenile (J2s) mobility inhibition at 5 $mgmL^{-1}$ of both water and methanolic crude extracts. Under greenhouse conditions, results showed a significant (p<0.01) inhibition of 97% and 89% on J2s penetration/establishment on tomato roots, with 100% and 50% root gall development inhibition on seedlings treated with *V. colorata* and *C. maxima* methanolic crude extracts, respectively. Furthermore, *V. colorata*, *S. lancea* and *C. sylvaticus* methanolic crude

extracts significantly inhibited the microbial activity of the tested rhizobacteria with minimum inhibition concentration (MIC) values ranging between 0.06 to 0.13, 0.01 to 0.12 and 0.12 μgmL^{-1}, respectively. A greenhouse study was conducted to evaluate the effect of powdered plant material of the eight botanicals at 0, 2, 6 and 10 g on nematode infested tomato seedlings arranged in a randomised complete block design with six replicates. At 56-days after the initiation of treatments, botanicals significantly ($p \leq 0.05$) reduced final nematode population densities, however they had no effects on chlorophyll and carotenoid content, dry shoot mass, dry root mass, and plant height and stem diameter.

The *in vitro* screening of plant-growth promoting properties of *B. licheniformis* ATCC 12752 and *P. fluorescens* ATCC 12525 indicated some plant growth attributes such as ammonia, IAA, siderophore, ethylene, hydrogen cyanide production and phosphorus solubilization. However, from the *in vitro* screening studies, both bacterial species lack the ability to produce protease enzymes. Furthermore, comparable to *P. fluorescens* ATCC 12525, *B. licheniformis* ATCC 12752 recorded the highest total flavonoids and phenolic content of 74.83 mg CE/g and 44.96 mg GAE/g, respectively, with DPPH radical scavenging IC_{50} of 191.08 μgmL^{-1}. The greenhouse results further showed that *B. licheniformis* ATCC 12752 and *P. fluorescens* ATCC 12525 applied separately significantly reduced plant growth of tomato seedlings, while as co-treatments the bacterial species stimulated tomato plant growth variables except dry shoot weight. Additionally, relatively to other treatments, *P. fluorescens* ATCC 12525 stimulated chlorophyll a+b and carotenoid content, while *B. licheniformis* ATCC 12752 significantly increased carbohydrate levels. The protein contents in both roots and leaves were significantly different among the treatments and untreated control.

This greenhouse study assessed eight different botanicals at 2, 6 and 10 g application rates and further evaluated the efficacy of the same plant species combined with *B. licheniformis* (*Bl*) ATCC 12752 or *P. fluorescens* (*Pf*) ATCC 12525 on vegetative growth, phytochemical accumulation in tomato seedlings, and their ability to manage *M. incognita* population density. At 56-days after initiating the treatments, *C. maxima, C. sylvaticus* and *M. plumbea* at highest application rate (10 g) significantly (p<0.05) reduced nematode numbers on the tomato roots and stimulated chlorophyll content, plant fresh and dry weight, plant height, root length and stem diameter. Furthermore similar results were observed on plants treated with *C. maxima, C. sylvaticus* and *M. plumbea* combined with either *B. licheniformis* or *P. fluorescens*. The final nematode population densities were significantly suppressed in plants treated with *C. maxima+Bl* with an RF value of 0.30. *Cucurbita maxima*, *P. sidoides*, *C. sylvaticus, S. aculeastrum* and *M. plumbea* combined with *B. licheniformis* significantly stimulated plant growth variables of tomato seedlings and similar results were noted on plants treated with the same botanicals combined with *P. fluorescens*. Consequently, an increase in total chlorophyll and carotenoid content was also noted with the same treatments. On the other hand, total protein and carbohydrate content were higher in plants treated with botanicals and *P. fluorescens*. Relative to the uninoculated tomato plants, the levels of flavonoid and total phenolic content increased in the untreated control by 130 and 19%, respectively. Therefore, the results of this current study suggest that the combination of botanicals with either *B. licheniformis* or *P. fluorescens* exhibit a synergetic relationship leading to juvenile penetration inhibition and resulted in reduced gall formation on tomato roots.

Furthermore, *P. sidoides* seedlings treated with either ground or crude extracts of *C. maxima* seeds significantly enhanced plant growth and reduced nematode infection. In addition, *C. maxima* seed extracts significantly increased the chlorophyll a+b and carotenoid content which might have enhanced the photosynthetic capacity of the plants at 10 g and 5 mg/mL. A reduction in total phenolics (68 and 64%), superoxide dismutase (1.33 and 1.45-fold) and carbohydrate (1.07 and 1.16-fold) content was recorded for both ground (10 g) or crude (5 $mgmL^{-1}$) extracts, whereas, a significant enhancement (10 g; 1.02-fold) and reduction (5 $mgmL^{-1}$; 1.06-fold) in total protein content was noted when compared to the untreated control. Notably, of all the botanicals, *C. maxima* and *M. plumbea* were more efficacious and could be further developed for use on *P. sidoides* production in soils with high *M. incognita* infestation.

The nematicidal effect of some of these botanicals alone or in combination with the rhizospheric bacteria denoted a promising management strategy that could easily be adopted against root-knot nematodes. Out of the eight botanicals tested *C. maxima* seeds exhibited good nematicidal properties with good egg hatch and juvenile mobility inhibition which could be due to the bioactive compounds such as octadec-9-enoic acid z (cis), octadecanoic acid, 17 octadecynoic acid, octanoic acid, dodecanoic acid, tetradecanoic acid, pentadecanoic acid, hexaonoic acid, 1-octanol, 1-octanal, 2,4-decadienal, (E-E) and 2-decenal, (E) identified with the use of Gas Chromatography-Mass Spectrometry (GC-MS) analysis.

CHAPTER 1: General introduction

1.1. Introduction

Nematodes are microscopic worms that are regarded as one of the most abundant species on planet earth, with estimates suggesting that about 3000 individuals can be present in just one hundred grams of garden soil (**Reynolds et al., 2011**). Generally, nematodes are chemotactic and require films of water for movement (**Neher, 2010; Janion-Scheepers et al., 2016; Reynolds et al., 2011**); however, they are found in both aquatic and terrestrial habitats. The family of nematodes consists of beneficial (which supports plant health) and parasitic (which parasitize plants, thereby causing stunted growth or total crop losses) species (**Jairajpuri and Ahmad, 1992**). Several nematodes are found at three trophic levels of the soil food web (**Freckman and Caswell, 1985**). At the first trophic level, nematodes feed on plants and algae, whereas they feed on bacteria and fungi at the second trophic level. Lastly, at the third trophic level, nematodes feed on other nematodes, and this level is considered to be the higher trophic level (**Freckman and Caswell, 1985; Mikola and Setälä, 1998; Franzluebbers, 2006; Kudrin et al., 2015**).

Consequently, there are more than 100 species of nematodes of agricultural and economic importance, with root-knot nematodes (RKN) belonging to the genus *Meloidogyne* being on top of the list. *Meloidogyne* species are ubiquitous plant endoparasites responsible for the vast majority of damages to arable crops, with global estimates of annual losses in the range of several billions of dollars. Yearly crop losses resulting from RKNs are estimated at between 80 to 157 billion dollars (**Singh and Kumar, 2015; Singh et al., 2015**). RKNs are incredibly polyphagous. Hence they parasitize more than 5000 species of higher plants in a wide range of

geographical distribution (**Jones et al., 2013; Abad et al., 2003**), most of which are cultivated plants.

Meloidogyne species are of particular interest within tropical and subtropical regions due to their extensive array and distribution of host species (**Cunha et al., 2018**). Globally, there are four *Meloidogyne* species of economic importance, including *M. javanica*, *M. incognita*, *M. arenaria* and *M. hapla* (**Loubser, 1988; Moens et al., 2009; Seid et al., 2015**). However, in South Africa, *M. javanica* and *M. incognita* are the most widely distributed and dominant *Meloidogyne* species (**Rashidifard et al., 2018**), with *M. javanica* being the most aggressive species (**Abebe et al., 2015; Sharma and Singh, 2016; Agenbag, 2016**). These root-knot nematodes invade plant roots in search of food and to complete their life cycle. When parasitism occurs, giant cells are formed due to feeding that leads to the formation of galls. These disrupt the translocation of photoassimilates to root tissues and absorption of water and nutrients from the soil (**Carneiro et al., 2005; Mitkowski and Abawi, 2003**). **Lamberti** (**1979**) reported a 50% reduction to total crop failure of watermelon (*Citrullus lanatus* Thunb.) yields. In other crops such as dry beans, a 45 to 90% crop loss has been reported (**http://ipm.ucanr.edu/PMG/r52200111.html**). Cereal cyst nematodes (*Heterodera* species) cause losses ranging from 30 to 100% in a wheat field, rice field yield loss of up to 80% caused by *Meloidogyne graminicola,* maize yield losses of up to 60% due to infestation by needle nematode *Longidorus breviannulatus* and up to 100% yield losses in *Ipomoea batatas* fields caused by the stem nematode *Ditylenchus destructor* (**Bernard et al., 2017**).

The usual method of control and management of RKN population density is based on the use of synthetic nematicides (**Haydock et al., 2006; Lima et al., 2018**). Continuous use of synthetic chemicals adversely affects the entire agricultural industry due to their toxicity, environmental and human health risks, and there are calls to limit their use (**Damalas and Eleftherohorinos, 2011**). During the manufacture and application of agrochemicals, tons of toxic chemicals and compounds released into the environment leach into groundwater, causing contamination, or getting volatilized and polluting the air, thus adversely affecting both non-targeted micro- and macro-organisms. They also contribute to global warming through greenhouse gas emissions **(Heimpel et al., 2013)**. For instance, pesticide manufacturing processes account for about 3% of the 100-year Global Warming Potential **(Audsley et al., 2009)**. Like most agrochemicals, nematicides are expensive; hence they increase the overall cost of crop production, and they are also a subject of environmental pollution concerns due to their toxicity and other adverse side-effects on the environment **(Onkendib et al., 2014)**. Globally, the continuous withdrawal of synthetic nematicides from agrochemical markets has left large-scale farmers with limited alternatives to manage RKNs population densities. In South Africa, emerging and small-scale farmers are the most affected by the use of synthetic chemicals due to their often high cost (**Khapayi and Celliers, 2016**).

The use of synthetic chemicals is a significant issue in developed countries and an escalating problem in many developing countries (**Ecobichon, 2001; Özkara et al., 2016**). According to the International Panel of Climate Change (**IPCC, 2013**), agriculture is viewed as one of the primary sources of emitted greenhouse gases (GHGs) such as nitrogen oxide (N_2O) and methane (CH_4) as described by **Pathak et al.** (**2014**). In both developed and developing countries,

synthetic chemicals are being applied at high rates, releasing carcinogens and other harmful substances which have a destructive impact on human and environmental health (**Özkara et al., 2016**). In recent years, the use of synthetic pesticides has increased from 4 to 5.4%, in both developed and developing countries (**http://www.fao.org/3/y3557e/y3557e11)**. This trend is alarming, but it is possible that, in the near future, the use of synthetic pesticides could diminish as a result of new legislation and precise assessments, as well as the growing worldwide demand for safe food, which will safeguard biodiversity and help peasant farmers. Moreover, as society demands more organic products, farmers are inclined to adopt the practice of smart agriculture to meet these requirements; this includes using biological controls, organic fertilizers, resistant cultivars, and ecologically safe methods of integrated pest management (IPM) (**http://www.fao.org/3/y3557e/y3557e11**).

Studies have shown that biological nematicides consist of several ranges of phytochemicals, which play a massive role as attractants and repellents of RKNs. Several plants such as neem, wild watermelon, wild cucumber, custard beans, and marigold have been shown to have nematicidal properties against RKNs (**Ploeg, 1999; Ntalli et al., 2011; Thies et al., 2016; Mashela et al., 2017**) when used in crop rotation regiments, and as soil amendments, rootstocks, and trap crops. These nematicidal properties have been reported to be due to allelochemicals, also known as secondary metabolites or natural products (**Latif et al., 2017; Hernández-Carlos and Gamboa-Angulo, 2019**). Plants or micro-organisms release certain chemical compounds into their environment, causing a direct/indirect inhibitory or stimulatory effect on other plants/micro-organisms through a mechanism known as allelopathy (**Rice, 1987**). Several allelopathic compounds have been studied extensively and have been shown to be responsible

for inhibiting herbivore attacks (**War et al., 2012; Cheng and Cheng, 2015**). Plants do not require these compounds to achieve primary activities such as plant growth and development (**Gebashe et al., 2020**). However, they are produced in different plant parts as secondary metabolites and are mainly used as a protective strategy against predators (**Pagare et al., 2015**).

1.2. Rational of the study/problem statement

Nematode surveys conducted by **Mtshali et al.** (**2002**) and **Ntidi et al.** (**2012**) revealed that South African soils are heavily infested with RKNs. Additionally, the withdrawal of synthetic nematicides such as methyl bromide and aldicarb has left agricultural lands exposed to RKNs, and this has presented a constraint for successful crop production due to high yield losses. Alternative management methods such as the use of bionematicides have gained a lot of interest in the past few years, even though the phytotoxicity of some bionematicides has continuously been reported (**Mashela et al., 2015; 2017**). The Botanical Society of South Africa encourages awareness about the conservation of indigenous plants in southern Africa through indigenous gardening to reduce the extinction of individual indigenous plants. The national population density of *P. sidoides* in South Africa is dwindling due to excessive harvest from wild populations for commercial gains; hence the plant has been listed on the Red List as an "endangered species" (**de Castro et al., 2012**).

Several studies have shown that root-knot nematodes are a source of menace to many tomato (*Solanum lycopersicum*) cultivars. RKNs affect the growth, development, and the overall yield ranging from 25 to 100% (**Seid et al., 2015**) depending on climatic factors and management strategies. Sustainable cultivation measures tailored towards enhancing the yield of tomato

(**tomato.pdf (kzndard.gov.za)**) and conserving *P. sidoides* (**Moyo and Van Staden, 2014**) have been reported; however, they are limited to *P. sidoides* and tomato nematode-resistant cultivars in the open market (**Sithole et al., 2016**).

Therefore, the successful suppression of *M. incognita* populations on *P. sidoides* and tomato would encourage the use of bionematicides in the management of RKNs. Findings from this study will further be economically viable for small-scale farmers as a cheap RKN management strategy and stimulations for wealth and job creation, which are in line with the National Development Plan of South Africa. Therefore, the study proposes to investigate the impact of bionematicides on the physiological growth and development of tomato and *P. sidoides* DC.

1.3. Aims and Objectives

Plant-parasitic nematodes (PPNs) negatively affect plant growth and development and therefore result in economic crop losses. Due to the growth limiting implications posed by RKNs infestations to a wide range of crops, different management tactics were established, and selected plants were screened to enhance and ensure sustainable production of both edible and non-edible crops. To successfully investigate whether the eight selected medicinal plants may be useful as bionematicides, a series of empirical trials were conducted to garner relevant information that will contribute to bridging the scientific knowledge gaps on the use of plant extracts/secondary metabolites from South African medicinal plants in the management of plant-endoparasitic nematodes. The information provided by these trials is intended to enhance the use of medicinal plant extracts because these plants are readily available, cheap, and easy to use. Furthermore, scientific findings could be used to develop RKN management approaches that can be easily

integrated into crop cultivation strategies and practices. Findings from this study will further be economically viable for small-scale farmers as a cheap RKN management strategy. Therefore, the goal of this study was to screen the nematicidal efficacy of *Cucurbita maxima* Duchesne, *Prunus africana* (Hoof. f.) Kalkman, *Pelargonium sidoides* DC., *Croton sylvaticus* Hochst, *Solanum aculeastrum* Dunal, *Vernonia colorata* (Willd) Drake, *Searsia lancea* (L.F.) F.A. Barkley and *Merwilla plumbea* (Lindl.) Speta, extracts on *Meloidogyne incognita* and evaluate their effects on the physiological growth of the nematode susceptible plants *P. sidoides* and *Solanum lycopersicum* L. with and without rhizobacteria, through the following objectives;

• To determine the phytochemical content and antioxidant activity of *P. sidoides*, *C. maxima*, *P. africana*, *C. sylvaticus*, *S. aculeastrum*, *V. colorata*, *S. lancea,* and *M. plumbea* and assess their mutagenic activity;

• To evaluate the nematicidal activities of methanol and water extracts of *P. sidoides*, *C. maxima*, *P. africana*, *C. sylvaticus*, *S. aculeastrum*, *V. colorata*, *S. lancea* and *M. plumbea* on *Meloidogyne incognita* egg hatching and juvenile mortality under *in vitro* and test their antibacterial (rhizospheric bacteria) activity;

• Evaluation of the individual and synergistic effect of microbal inoculation (either *Bacillus licheniformis* or *Pseudomonas fluorescens*) and powdered botanicals on the performance of nematode-infested *S. lycopersicum* under greenhouse condition; and

• To compare the application methods (drenching and mulching) of the eight selected botanicals on the growth of nematode infested *Pelargonium sidoides* under greenhouse conditions and determine the putative bioactive nematicidal compound (s) from *C. maxima* seeds using GC-MS analysis.

1.4. Thesis outline

The thesis consists of seven Chapters as detailed and described herein; a detailed description of the research problem (Chapter 1) followed by a comprehensive literature review (Chapter 2). Then, each of the four subsequent chapters (Chapter 3, 4, 5 and 6) addresses each of the four objectives. Chapter 7 consists of abridged findings of all Chapters to provide general conclusions and recommendations concerning future research. The citation and references reported are as per the guidelines of the South African Journal of Botany.

CHAPTER 2: Literature review

2.1. Introduction

Nematodes are microscopic, non-segmented, thread-like roundworms that are among the most abundant and cosmopolitan group of metazoans that inhibit a wide range of habitats and ecosystems (**Seesao et al., 2017**). Nematodes inhabit both terrestrial and aquatic (marine and freshwater) ecosystems (**Bernard, 1992; Tahseen, 2012; Majdi and Traunspurger, 2015**). Their morphological presentation consists of a worm-like shape of about 1 mm in length with a flexible and resilient exoskeleton (**Reynolds et al., 2011; Ravichandra, 2014; Seesao et al., 2017**). Nematode families comprise of both beneficial free-living species and harmful parasitic species. Free-living nematodes play an essential role in the decomposition of organic matter in soils, thus contributing to the release and recycling of mineral nutrients in the environment. At the same time, parasitic forms are mainly significant because of their role as disease-causing agents in plants, animals, and humans (**Seesao et al., 2017**). Plant-parasitic nematodes are categorized into three types: sting, lance, and gall nematodes (**Handoo, 1998**). Root-knot or gall nematodes are the most damaging parasitic nematodes (**Handoo, 1998**). Although root-knot nematodes are nearly microscopic underground dwelling organisms and are not noticeable to growers, the galls they induce, which present as a swollen bump on the roots of plants, are quite large and are visible. The presence of galls on the roots prevents proper absorption of mineral nutrients and water from the rhizosphere, which leads to poor nourishment of plants and reduced overall crop yield. Many researchers cite these worms as the hidden enemy of growers (**Sasser, 1989; Cooke, 1990; Gokte-Narkhedkar et al., 2006; Singh and Phulera, 2015**) due to their parasitism on host plants. Root-knot nematodes (*Meloidogyne* spp.) are considered the most aggressive plant-parasitic nematode species and are economically one of the most important

pests that can potentially constrain crop production (**Jones et al., 2013; Bernard et al., 2017; Coyne et al., 2018**). They cause growers and horticulturalists estimated annual yield losses of approximately 125 million US dollars, excluding the damage they cause on other non-economical field crops, including home gardens and non-edible plants such as ornamentals.

In most cases, the damages they cause go unnoticed because the extent of harm caused by nematodes is ascribed to other causes related to abiotic and biotic effects on plants (**Lambert and Bekal, 2002**). Out of more than 100 identified *Meloidogyne* species, the four most destructive species include *Meloidogyne javanica*, *Meloidogyne arenaria*, *Meloidogyne halpa* and *Meloidogyne incognita* (**Jones et al., 2013; Bernard et al., 2017**). When not managed properly, they can cause significant losses in crop yield, sometimes resulting in total crop failure. Globally, nematodes are estimated to account for about 12.3% of crop yield losses per annum (**Singh et al., 2015**). *Meloidogyne javanica* and *M. incognita* are the most predominant root-knot nematodes in most South African agricultural production areas that often consist of various plant hosts (**Fourie et al., 2015; Mbatyoti et al., 2021**).

The expansion of the commercial production of many foods and cash crops is limited due to the abundance of root-knot nematodes and limited ability to manage plant-parasitic nematodes. This has been partly attributed to the build-up of nematodes on arable fields due to restrictions or the withdrawal of synthetic nematicides from agrochemical markets due to the potential toxicity they impose on humans and the environment (**Warnock et al., 2017**). Hence, this has resulted in the limited availability of registered nematicides from the agro-markets. The withdrawal of synthetic nematicides from agrochemical markets has occurred due to the damage they cause on

biodiversity and non-target organisms, among other factors (**Bernardes et al., 2015**). This has resulted in a call for the implementation of sustainable and ecologically friendly alternatives. Bio-alternatives, such as the use of plant-derived nematicides or bionematicides, have gained a lot of interest amongst many researchers since their products are safe and efficiently biodegraded. However, phytotoxicity still hinders the deployment of bionematicides, therefore, complicating the management of root-knot nematodes (**Mashela et al., 2015; Sithole, 2016; Mashela et al., 2017**).

2.2. Morphology and life cycle of root-knot nematodes

Root-knot nematodes are described as cylindrical unsegmented worms that taper towards the head and tail. Depending on the species, stage of development, and sexuality, they vary in size and morphology. The body length of adult males ranges between 791 to 1660 μm, females usually between 419 to 950 μm while infective juveniles have body lengths ranging between 200 to 460 μm (**Coetzee, 1956; Whitehead, 1969; Kaur and Attri, 2013; Archidona-Yuste et al., 2018)**. The mature female nematode of the genus *Meloidogyne* is characterized by a loss of its worm-like shape and becomes pear-, kidney- or lemon-shaped (**Coyne et al., 2007**). In comparison to higher animals, root-knot nematodes contain all the critical organ systems except the circulatory and respiratory organs (**https://mrec.ifas.ufl.edu/lso/SCOUT/Nematodes.htm**). Their body consists of a transparent covering or exoskeleton known as the cuticle, making it easy to observe their inner parts for species identification (**Bernard et al., 2017; Shah and Mahamood, 2017**). The body of root-knot nematodes is a typical "tube within a tube" structure in which the inner alimentary canal consists of a digestive tract and gonad, which are surrounded by the outer body wall, which bears a series of dorsally and ventrally placed longitudinal muscles

that are attached to the hypodermis (**Bernard et al., 2017**). Dorsal and ventral nerves control the contractions of these muscles for locomotion. A cavity derived from the blastula known as pseudocoelom is filled with fluid (**Decraemer and Hunt, 2006; Bernard et al., 2017**), a fluid that shelters the reproductive system and other organs. This fluid provides turgor pressure, which sustains the body shape, and serves in the circulation of nutrients and gaseous exchange (**Bernard et al., 2017**).

Root parasitic nematodes possess a hollow mouth spear (a hypodermic needle-like) called a stylet at the anterior end/head. The stylet is the primary infection structure, and it is adapted for piercing and sucking (**Singh and Phulera, 2015; Bernard et al., 2017**). When the stylet is injected into the plant host, it punches through the plant cell using a parasitism mechanism and withdraws food and useful metabolites from its host (**Bernard et al., 2017**). The stylet is linked to the pharynx (esophageal region), atypical tylenchoid esophagus, metacorpus, a narrow isthmus and a posterior glandular basal bulb (**Kaur and Attri, 2013; Bernard et al., 2017**). The metacorpus serves to transfer enzymes required for primary infection and facilitates the movement of plant nutrients into the intestine. The esophagus is connected to the intestine, while the intestine ends at the rectum and cloacae in the female and male nematode**,** respectively (**Singh and Phulera, 2015**). Reproductive structures in females include ovaries, seminal receptacles, a uterus, an ovijector, and a vulva. In contrast, in males, the reproductive system consists of a testis, a seminal vesicle, and a vas deferens opening into a cloaca (**Bernard et al., 2017**).

Nematodes are endoparasites; their life cycle is completed inside their host plant, which serves mainly as a source of nourishment and provides a safe haven for reproduction (**Caillaudet al., 2008**). The life cycle of *Meloidogyne* spp. consists of six stages that include the egg stage, four juvenile stages (J1, J2, J3, and J4), and an adult stage (**Lambert and Bekal, 2002; Osunlola and Fawole, 2014**) (**Figure 2.1**). The eggs are enclosed in a protective jelly-like egg mass outside the surface of galled roots. Briefly, embryogenesis occurs, and the first-stage juvenile (J1) molt takes place within the egg. Therefore, when the egg hatches, the emerging individual is a second-stage juvenile (J2). It is migratory (**Bernard et al., 2017**) and the only infective stage considered vermiform. In order to survive, J2s are well equipped for parasitism (**Perry, 1977**). Infective J2 individuals move freely in the soil and are attracted to root tips by root exudates of host plants (**Reynolds et al., 2011; Teillet et al., 2013; Bernard et al., 2017**). They invade the elongation zone of roots using cell wall hydrolytic enzymes such as cellulases, endoglucanases, endoxylanases, and pectate lyases, secreted into the apoplast from their esophageal or subventral glands (**Abad et al., 2008; Perry and Moens, 2011; Bernard et al., 2017**).

After root invasion, an intercellular system is formed throughout the aerenchymatous tissues of the cortex as the establishment of a feeding site, and giant cells occur concurrently (**Lambert and Bekal, 2002**; **Massawe, 2014**). The galls in the vascular system are formed as the plant responds to nematode parasitism (**Bridge et al., 1982**). In the absence of host roots, *Meloidogyne* spp. undergo several biochemical and physiological adaptations by delaying the embryogenesis phase, quiescence, and diapauses, through feeding on its lipid reserves; this prolongs its viability and ensures its survival until host roots are available (**Moens et al., 2009**). Thus, after the eggs hatch, juveniles emerge and invade the host roots (**Perry et al., 2009**). After establishing a

permanent feeding site and becoming sedentary, generally in the vascular cylinder (**Palomares-Rius et al., 2017**), J2s undergo multiple morphological alterations and molt into third-stage juveniles (J3), then fourth-stage juveniles (J4), before advancing into either female or male adult nematodes (**Abad et al., 2003;Thapa, 2018**).

The RKNs males are vermiform and, in most cases, exhibit a short life span because *Meloidogyne* spp. are primarily parthenogenetic. Thus, the males are not necessary for fertilization and reproduction. However, in other cases, males leave the root to copulate with females for breeding, while the female remains intact in the roots (**Lambert and Bekal, 2002**). Sexual dimorphism is therefore associated with lifestyle, with the females being sedentary while males are vermiform and motile (**Castagnone-Sereno et al., 2013**). The female nematode becomes more substantial in size as it matures and begins producing eggs outside the roots (**Lambert and Bekal, 2002**). Adequate temperature, moisture, and food availability are the major factors that enhance egg hatching (**Morris et al., 2011**). A female nematode may lay 30 – 50 eggs per day during its life cycle, which generally takes 21 – 28 days to be completed, with a possibility of eight generations per year at 30ºC (**Massawe, 2014**).

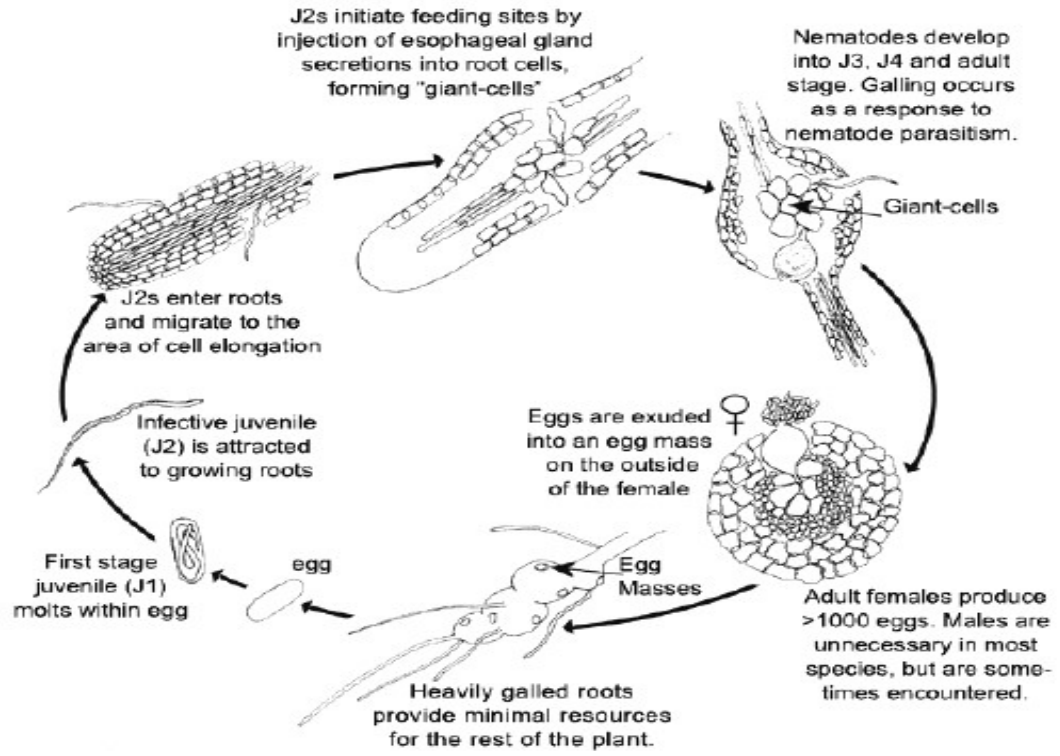

Figure 2.1. The life cycle of root-knot nematode (*Meloidogyne* spp.) (**Singh and Phulera, 2015**).

2.3. Effect of root-knot nematodes on plant growth and yields

Several crop species are susceptible to RKNs and can be severely affected. Globally, the recorded number of susceptible plants to RKN is very high and is continuously increasing, with more than 2000 species reported to be vulnerable to RKNs (**Sasser, 1980; Abad et al., 2003; Khalil, 2013a; Khalil, 2013b; Bernard et al., 2017**). The extent of the damages caused by RKNs is more severe because parasitic nematodes can promote plant susceptibility to other parasites (**Bridge et al., 2005**). Juveniles proceed by infecting the roots of their host and undergo morphological changes. Feeding sites are established by nematodes, consisting of root galls that perturb nutrient/mineral assimilation and the translocation of photosynthates; thus, plant development is suppressed (**Escobar et al., 2015**). Root-knot nematodes reduce the root-to-shoot

ratio, affecting photosynthesis and yields (**Abbasi and Hisamuddin, 2014**). The standard warning signs associated with the invasion of RKNs include yellowing of leaves, a reduction in the root system, root structure distortion, or an increase in root diameter due to gall formation and eventually the withering of plants resulting in reduced plant productivity (**Agrios, 1997**).

2.4. Methods used to manage plant-parasitic nematodes

Many farmers have adopted integrated pest management (IPM) strategies to solve everyday agricultural problems concerning pest management. This pest management system is used to enhance the effectiveness of other pest control approaches and reduce the frequent use of pesticides in a bid to avoid pesticide resistance by various organisms. The use of chemicals as part of a pest management strategy is considered normal. Globally, growers rely on synthetic chemicals to provide protection against insects, weeds, diseases and stimulate plant growth and yield. Soils are fumigated before planting to provide a microbial pathogen-free environment. However, the application of synthetic chemicals comes with significant adverse effects due to the broad spectrum of action of most pesticides; thus, they mediate alterations in ecological balance, pose threats of environmental contamination, and sometimes have residual toxic effects on the food-chain (**Warnock et al., 2017**). They also show inadequate or inconsistent results, and pests may develop long-term resistance when pesticides are used consistently (**WHO-FAO, 2018**).

Consequently, for the past few decades, the use of synthetic chemicals (such as methyl bromide) has been steadily declining due to its toxicity and associated efforts aimed at mitigation of climate change **(WHO, 2003)**. This, and other factors, has led to a shift towards developing new

alternatives within the legal structure of organic farming. In most cases, the conventional methods of plant protection still include the extensive use of chemicals in IPM.

2.4.1. Crop rotation with the correct cultivars

Crop rotation is defined as "*the practice of growing different crops in succession on the same land chiefly to preserve the productive capacity of the soil*." Soil characteristics such as the physical, biological, and chemical nature can reduce the damage exerted by PPNs (**Westphal, 2011**). Hence, crop rotation is employed to maintain soil fertility and achieve appropriate soil health. Growing different crops from different families in succession has been shown to control pest populations below levels that cause economic damage (**Skellern and Cook, 2018**). This is partly because crop rotation can easily interrupt pest life cycles (**Skellern and Cook, 2018**). This strategy has been used for many years, although some researchers indicate that using one management tactic is not sufficient. Hence they propose the incorporation of new approaches, along with existing strategies, which are more effective.

However, **Westphal** (**2011**) observed that rotation with soybean and corn was not effective at suppressing the impact of *M. incognita* on watermelon in southern Indiana in a designed production system. The author further suggested that such a system can be modified using an integrated management approach. Shortage of arable land is a significant constraint to the extensive application of crop rotation, especially in farming areas where intensive cropping is practiced and where susceptible crops are the main cash crops. Some studies have shown that population densities of *M. incognita* can be suppressed by producing crops with genotypes that exhibit resistance traits (**McSorley and Dickson, 1995; Wang et al., 2002; Ortiz et al., 2015**).

For example, instead of using small grains as cover crops, plants that exhibit resistance to *M. incognita* and/or bio-fumigation potential and serve as cover crops can be selected and used. Crop rotation involves regular tillage and is also associated with a lack of permanent vegetative cover; thus, this practice exposes fields to frequent soil disturbances, which is a major contributing factor in soil degradation. However, **Massawe** (**2014**) reported that the root-knot nematode population tends to decline in numbers in the soil where tillage occurs frequently. Similar remarks were made by **Bailey and Lazarovits** (**2003**) when they reported that the life cycle of soil-borne pathogens could easily be disturbed by everyday agricultural practices such as weeding and tillage.

On the other hand, extensive research has shown that the efficient use of crop rotation successfully manages various nematode species with a narrow host array. Management of PPNs is a challenge, especially in soils heavily infested with a range of nematode species or where susceptible host plants are sequentially grown (**Weatphal, 2011**). Rotation of crops involving non-hosts provides enough time to allow the nematode population to drop below the damaging economic threshold before host plants are planted; rotation is mainly used in cases of soil fertility restoration and to reduce soil degradation. The main goal of alternating crops is to reduce PPN's population densities to acceptable levels by planting a resistant or non-host plant followed by a tolerant plant before growing a host plant (susceptible) (**Table 2.1**). In order to reduce the population of a pest or disease, it is best to rotate crops from different families and crops that are not susceptible to the same pathogens. **Guerena** (**2006**) highlighted that rotation between pumpkin and cucumber would not be effective since both crops belong to the Cucurbitaceae family.

Additionally, although cucumber and tomato belong to different families, they cannot be used on the same rotation following each other, given that both crops are host plants to *Meloidogyne* spp. (**Guerena**, **2006**). The population of root-knot nematodes can be reduced by rotating with crops that are known to be poor hosts. Sweet corn is considered a poor host; hence, it is an ideal candidate for crop rotation, particularly in areas where root-knot nematodes have exerted severe damage (**Table 2.1**). Other poor hosts that can be considered include garlic, asparagus, shallots, and onions. Furthermore, it has been reported that cool-season crops, including Irish potatoes, radishes, broccoli, greens, and cabbages, are less likely to develop a significant reduction in crop yields when infected by root-knot nematodes, even though they fall within the susceptible group of plants. This feature is attributed to the fact that they grow best in cooler climatic conditions, which are unfavorable for the multiplication of root-knot nematodes (**López-Perez et al., 2010**).

Moreover, information extracted from **https://plantdiseasehandbook.tamu.edu/** highlighted that nematodes pave the way for soil-borne pathogens to infect the host plant; in most cases, root rot and wilt diseases are experienced. For instance, a bean variety resistant to infection by the *Fusarium* wilt pathogen will become susceptible to the same pathogen if infected with root-knot nematodes. Thus, it is preferable to choose bean varieties that are resistant to pathogens in a root-knot nematode infested field with a history of *Fusarium* wilt.

Table 2.1. Crop grouping for rotation to control soil-borne diseases (source https://plantdiseasehandbook.tamu.edu/problems-treatments/methods-and-materials/non-chemical-control-of-plant-diseases-in-the-home-garden/).

Group A	Group B	Group C	Group D	Group E	Group F
Cantaloupe, Cucumber, Honeydew Melon, Pumpkin, Squash, Watermelon.	Brussels sprouts, Cabbage, Cauliflower, Collards, Lettuce, Mustard, Radish, Rutabaga, Spinach, Swiss chard, Turnip.	Eggplant, Irish potato, Okra, Pepper, Tomato.	Beet, Carrot, Garlic, Onion, Shallot, Sweet potato.	Sweet corn	Bean, Cowpea, Pea.

2.4.2. Fallowing

Fallowing involves leaving a piece of land idle or with no cropping activities for a defined period, with an intention to reduce soil pathogen populations and to enhance beneficial soil micro-organisms. Studies have shown that in fallowed land, PPN population densities decline due to starvation due to the lack of host crop plants (**Adediran et al., 2005; Chen and Tsay, 2006**). Plant-parasitic nematodes can reproduce and multiply in favorable conditions, such as in the presence of host roots under specific temperatures and moisture, which permit their life cycle to be completed (**Lambert and Bekal, 2002; Pokharel, 2011**). However, **Lambert and Bekal** (**2002**) have highlighted that in the absence of host plants or during cold seasons, nematodes prolong the embryogenesis stage.

The population of organisms which prey on PPN (bacteria, fungi, and nematodes) escalates in less cultivated soils as compared to soils where cultivation occurs intensively. Other benefits associated with fallowing include weed and insect control. **Ferraz and Brown** (**2002**)

highlighted that growers often adopt fallowing to reduce PPN population densities by starving the nematodes, which occurs in the absence of host plants or food residues. Plant-parasitic nematode populations on fallow lands have been shown to decline rapidly to a point below the damage threshold (**Cadet et al., 2003; Chen and Tsay, 2006; Archidona-Yuste et al., 2018**). Fallowing is a soil-borne management strategy that is mainly used in the potato industry, where fields are deliberately left uncultivated to reduce the risk of economic losses due to soil pathogens. This system is primarily helpful when employed in months when soil temperatures are high with the possibility of low rainfall. Managing RKNs using fallowing has been shown to be most effective during the summer season when temperatures are elevated (**McSorley, 1998; Chen and Tsay, 2006; Cadet et al., 2003**).

Tillage practices may be adapted for the control of PPN populations. Several reports show that soil nematode population is significantly affected by tillage (**Lopez-Fando and Bello, 1995; Okada and Harada, 2007; Schmidt et al., 2017; Mashavakure et al., 2018**). It is also known that tillage affects soil surface micro-topology and bulk density, as well as thermal and hydro-properties (**Shen et al., 2018**). Thus frequent tilling of farmland keeps the soil dry, free of weeds, and exposes soil-borne disease-causing organisms, such as nematodes, to excess heat and drying through soil solarisation, which kills them (**Pokharel, 2011**). The composition of PPNs is significantly affected in soils with higher bulk density and more evenly distributed pore space (**Massawe, 2014**). Pore space distribution and bulk density are high in disturbed soils such as those where intense agricultural activities like regular tilling and cropping occur. Some studies reported that tillage may have little effect or increase some PPNs species populations, however, it was noted that no-tillage supported a higher density of some species of PPNs (**Lopez-Fando**

and Bello, 1995; Schmidt et al., 2017), thus suggesting that tillage effects on PPN population may be species-dependent.

2.4.3. Biological control

Biological control (BC) involves the use of living organisms to suppress other living organisms, i.e. pests. This system was implemented in 1956 by the International Organization of Biological Control, with a goal to enhance environmentally friendly, socially acceptable, and economically efficient methods to combat pests and diseases in the field of agriculture and forestry. This method has since been used for decades (**Colmenarez et al., 2018**). The use of BC involves the reduction of plant-parasitic nematode population and consequently enhances the natural enemies in the soil. The use of biological control is the only agricultural practice that can restrict the use of synthetic chemicals in integration. It is mainly practiced in organic farming, where production occurs on a small scale with limited use of land and/or under greenhouses or shade houses. Several soil microbial species have been identified to feed on parasitic nematodes (**Tian et al., 2007; Elhady et al., 2017; Elhady et al., 2018**). These microbial pathogens are commercially available and designed to control nematodes. *Bacillus* and *Pseudomonas* spp. belonging to the group of aerobic and endospore-forming bacteria are the most dominant rhizobacteria with excellent nematicidal properties against PPNs (**Table 2.2**).

Numerous *Bacillus* strain*s* are used extensively globally and have shown significant suppressive activities on PPN populations, thus promoting plant growth. **Abd-Elgawad** (**2016**) highlighted the significance of bacteria as biocontrol agents. Plant growth-promoting rhizobacteria (PGPR) are defined as root-colonizing bacteria that protect plants against pathogens and are essential in

managing PPNs (**Mhatre et al., 2019; Mhatre et al., 2020**). The rhizobacteria exhibit various modes of action with beneficial effects on plants through direct and indirect mechanisms (**Figure 2.2**). These include plant growth enhancement due to their ability to produce or induce phytohormone production and increase phosphorus and nitrogen availability via phosphorus solubilization and nitrogen fixation, and protect plants through the secretion of enzymes, toxins, and a broad class of bioactive metabolites that can regulate nematode behavior (induces mortality of nematode eggs and second-stage juveniles). Rhizobacteria may cause the production of repellents by the host plant, alter the development of the nematode feeding site, compete with nematodes for essential nutrients and enhance plant-induced systemic resistance (ISR) (**Siddiqui and Mahmood, 1999; El-Nagdiand Youssef, 2004; Suryadi et al., 2019**). **Bakengesa and Wesemael** (**2016**) evaluated the potential use of *Paenibacillus* spp. as a biocontrol agent against some RKNs, namely *M. fallax*, *M. chitwoodi*, *M. javanica*, *M. enterolobii*, *M. incognita* and *M. hapla* under *in vitro* and *in vivo* conditions using 10% and 100% bacterial suspensions (BS). The authors stipulated that *Paenibacillus* spp. elicited both nematistatic and nematicidal effects. They showed that at 100% BS, *Paenibacillus* spp. induced more than 90% J2 mortality and inhibited egg hatching by 97% to 99% on all plant-parasitic nematode species tested. In addition, *Paenibacillus* spp. was shown to be effective in controlling RKNs using different modes of action, including paralysis and antibiosis, as demonstrated by mortality, immobility, and inhibition of egg hatching and prevention of host root invasion (**Bakengesa and Wesemael**, **2016**).

As with any chemical or biological control agents, they must go through the evaluation processes before being registered and commercialized (**Arjjumend and Koutouki, 2018**). The legalization

of BC is a costly process, just like those of synthetic chemicals. However, the process is done to ensure that the end product presented is safe for humans and the ecosystem. The employment of BC to reduce the use of chemical control measures has proven to be substantially beneficial (**Alori and Babalola, 2018**). As a result, a broad range of BC formulated products is commercially available. Conventional farms are recognized for being specific when selecting different farming practices. The employment of biological pesticides is a promising alternative because they are comprised of beneficial microbes or products that can be used to control diseases and improve soil and crop health (**Alori and Babalola, 2018**). The use of biochemicals has intensified due to their low environmental impacts. For instance, it is less likely that pests would develop resistance to microbial biocontrol agents; hence, PGPR is essential in preventing and controlling nematodes.

Table 2.2. The efficacy of PGPR inoculation as a biocontrol agent on plant-parasitic nematodes (**Mhatre et al., 2019**).

PGPR strains Agriculture	**Crops**	**Nematodes**	**Reference**
Bacillus subtilis	Tomato	*Rotylenchulus reniformis*	**Niknam and Dhawan, 2001**
Azotobacter chroococcum	Tomato	*Meloidogyne incognita*	**Chahal and Chahal, 2003**
	Brinjal	*Meloidogyne javanica*	**Bansal and Verma, 2002**
Pseudomonas stutzeri	Turmeric	*Meloidogyne incognita*	**Seenivasan et al., 2001**
Pseudomonas fluorescens + oil	Citrus	*Tylenchulus semipenetrans*	**Reddy et al., 2000**

cakes			
Pseudomonas fluorescens and *Rhizobium leguminosarum*	Bean	*Meloidogyne javanica*	**Tabatabaei et al., 2017**
Bacillus subtilis	-	*Meloidogyne incognita*	**Adam et al., 2014**
Pseudomonas putida, Pseudomonas fluorescens, Serratia marcescens, Bacillus amyloliquefaciens, Bacillus subtilis and *Bacillus cereus*	Tomato	*Meloidogyne incognita*	**Almaghrabi et al., 2013**
Pseudomonas fluorescens	Banana	*Meloidogyne javanica*	**Rodrıguez-Romero et al., 2008**
Bacillus isolates		*Meloidogyne incognita, Heterodera cajani*	**Siddiqui and Shakeel, 2007**
Paenibacillus macerans		*Meloidogyne exigua*	**Oliveira et al., 2009**
Pseudomonas fluorescens		*Heterodera cruciferae*	**Aksoy and Mennan, 2004**
Bacillus sp.	Tomato and pepper	*Meloidogyne incognita*	**Kokalis-Burelle et al., 2002**
Bacillus velezensis and *Bacillus mojavensis*	Soybean	*Heterodera glycines*	**Xiang et al., 2017**
Paenibacillus polymyxa	Tomato	*Meloidogyne incognita*	**Son et al., 2009**

and *Paenibacillus lentimorbus*			
*Bacillus subtilis, Paenibacillus polymyxa*and *Burkholderia cepacia*	Tomato	*Meloidogyne incognita*	**Siddiqui and Akhtar, 2009**
Bacillus sp., *Azotobacter* sp., *Pseudomonas putida* and *Pseudomonas fluorescens*	Tomato	*Meloidogyne incognita*	**Anwar-ul-Haq et al., 2011**
Different PGPR	Tomato	*Meloidogyne incognita*	**Alfianny et al., 2017**
Pasteuria sp.	Cotton	*Rotylenchus reniformis*	**Schmidt et al., 2010**
Pseudomonas putida and *Pseudomonas alcaligenes*	Chickpea	*Meloidogyne incognita*	**Akhtar and Siddiqui, 2009**
Pseudomonas alcaligenes and *Bacillus pumilus*		*Meloidogyne incognita*	**Akhtar and Siddiqui, 2008**
Bacillus amyloliquefaciens	Tomato	*Meloidogyne incognita*	**Burkett-Cadena et al.,2008**
Rhizobium etli	Tomato	*Meloidogyne incognita*	**Reimann et al., 2008**
Pseudomonas oryzihabitans	Potato	*Globodera rostochiensis*	**Andreoglou et al., 2003**

Rhizobium etli	Potato	*Globodera pallid*	**Reitz et al., 2000**
Rhizobacterial strains	-	*Esocriconema xenoplax*	**Mota et al., 2017**
Bacillus enterobacter and *Pseudomonas* sp.	-	*Meloidogyne incognita*	**El-Sayed et al., 2014**
Bacillus sp.	Yellow melon	*Meloidogyne incognita*	**Medeiros et al., 2009**
Bacillus sp. and *Lysobacter* sp.	Tomato	*Meloidogyne incognita*	**Zhou et al., 2016**
Bacillus subtilis	Carrot	*Meloidogyne incognita*	**Rao et al., 2017**
Bacillus tequilensis and *Bacillus flexus*	Basil	*Meloidogyne incognita*	**Tiwari et al., 2017**
Nitrogen-fixing bacteria	Banana	*Meloidogyne incognita* and *Radopholus similis*	**Aggangan et al., 2013**
Streptomyces sp.	Eggplant	*Meloidogyne incognita*	**Rashad et al., 2015**
Streptomyces sp.	-	*Meloidogyne incognita*	**Ruanpanun et al., 2010**
Pseudomonas aeruginosa	Mungbean	*Meloidogyne* sp.	**Siddiqui *et al.*, 2001**
Pseudomonas aeruginosa and *Bacillus subtilis*	Tomato	*Meloidogyne javanica*	**Siddiqui, 2002**
Bacillus thuringiensis	-	*Meloidogyne incognita*	**Dhawan et al., 2004**
Pseudomonas striata and *Rhizobium* sp.	Pea	*Meloidogyne* sp.	**Siddiqui and Singh, 2005**

Pseudomonas fluorescens	Mungbean	*Heterodera cajani*	**Latha et al., 2000**
Pseudomonas fluorescens	Tomato	*Meloidogyne javanica*	**Eltayeb, 2017**
Pseudomonas fluorescens	Castor bean and Tomato	*Rotylenchulus reniformis*	**Poornima, 2015a**
Pseudomonas fluorescens	Tomato	*Meloidogyne incognita*	**Poornima, 2015b**
Pseudomonas fluorescens	Banana	*Helicotylenchus multicinctus*	**Selvaraj et al., 2014**
Different PGPR strains	Grapevines	*Meloidogyne ethiopica*	**Aballay et al., 2013**
Consortium: *Pseudomonas fluorescens* (Pf 128) and *Bacillus subtilis* (Bbv 57)	Tomato	*Meloidogyne incognita*	**Meena et al., 2012**
Bacillus polymyxa +VAM	Tomato	*Meloidogyne incognita*	**Liu et al., 2012**
Pseudomonas fluorescens	Jasmine	*Meloidogyne incognita*	**Seenivasan and Poornima, 2010**
Methylobacterium fujisawaense	-	*Meloidogyne incognita*	**Prabhu et al., 2009**
Pseudomonas fluorescens	Okra	*Meloidogyne incognita*	**Veronika and Khan, 2015**
Pseudomonas fluorescens and *Bacillus subtilis*	Rice	*Meloidogyne graminicola*	**Priya, 2015**

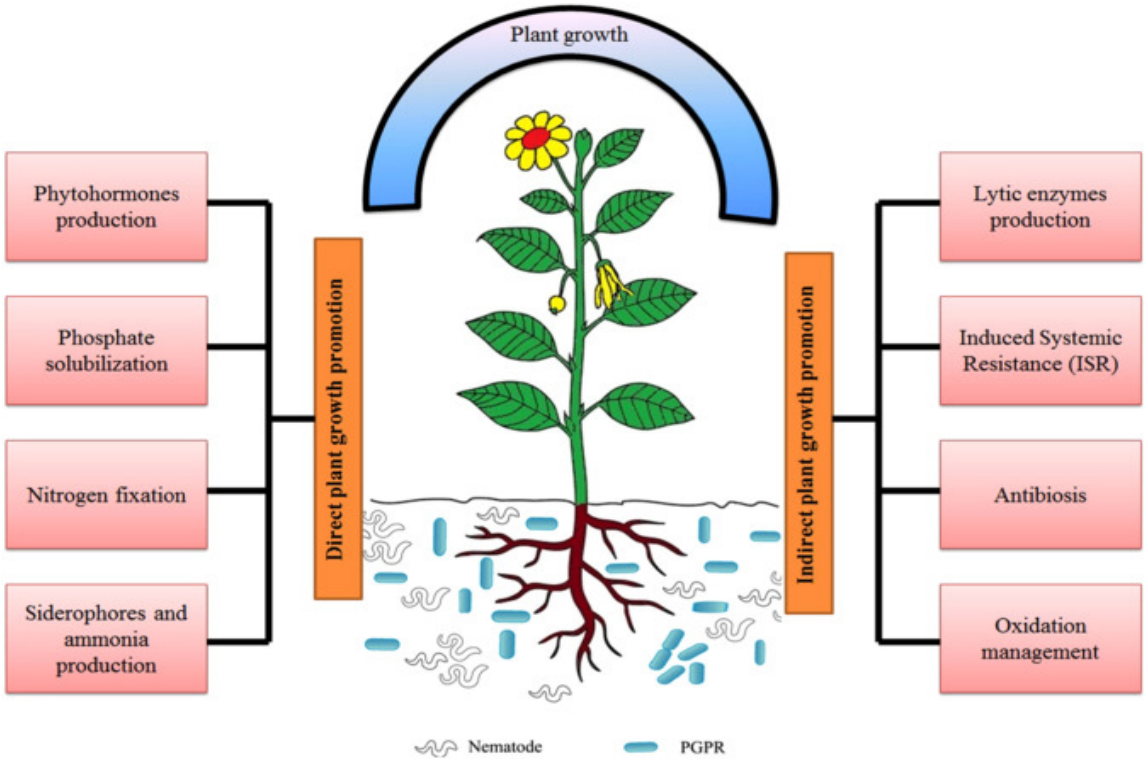

Figure 2.2. The influence of the PGPR pathway on the stimulatory effects of direct and indirect plant growth properties and the inhibitory attributes of nematodes (**Mhatre et al., 2019**).

2.4.4. Plant waste products as soil organic amendments

Amendments are mainly fresh or dry bio-products and wastes from industrial, agricultural, biological, and other activities (**Akhtar and Malik, 2000**). Soil amendments are made up of organic materials with properties that enable them to enhance soil fertility, improve soil structure and health (**Antonious, 2016**), increase plant resistance towards diseases, and with a high potential of releasing compounds that can be highly toxic to nematodes (**McSorley, 2011; Dutta et al., 2019**). This method has proven to be highly effective when compared to other methods due to availability, affordability, usability, lack of contaminants, and enrichment of the soil with organic materials (**Antonious, 2016; http://www.fao.org/3/a0100e/a0100e02.htm**).

Furthermore, soil amendments may also contain stimulants that are beneficial to microorganisms that act as nematode antagonists while at the same time enhancing plant growth and yield (**Parihar et al., 2012**). Frequent soil amendments used in the management of RKNs include animal manures, crop residues, and other concentrated organic amendments. These organic amendments can be applied to the soil in solid or liquid form. A study by **Xiao et al.** (**2016**) showed liquid organic manure could control RKNs and stimulate plant health in tomatoes. Regardless of the type of organic soil amendment, they are environmentally suitable for managing the population densities of plant-parasitic nematodes.

However, the system has its limitations, such as expensive transportation and large quantities needed per unit area, which leaves the strategy impractical in large scale farming (**Mashela, 2002; Mateille et al., 2007**). Furthermore, **Akhtar and Malik** (**2000**) highlighted that the mode of action of organic soil amendments in controlling plant diseases with stimulated beneficial micro-organisms is complex and varies with the type of soil amendment used. It is important to note that higher organic matter alone does not guarantee better control of root-knot nematodes. However, some reports suggest that higher organic matter ensures a better chance of developing antagonistic micro-organism populations within the soil (**Akhtar and Malik**, **2000; Rashid et al., 2016**). Fungi are essential antagonists because they function by trapping nematodes with their mycelium and use them as a food source (**Nordbring-Hertz et al., 2001; Devi, 2018; Moosavi and Zare, 2020**). The effectiveness of the organic matter is dependent on the type used. The best organic matter can be prepared by leaving the waste product of crops such as small grains or legumes after harvest. Furthermore, to improve crop production, nitrogen can be added because the organisms in the soil use nitrogen reserves to break down green manure crops.

2.4.5. Allelopathy and allelochemicals

Allelopathy is a phenomenon whereby secondary metabolites produced by living organisms influence the growth and development of other living organisms either positively, i.e. pesticides, herbicides, and fungicides, or negatively, i.e. auto-toxicity, soil sickness, and biological invasion. Naturally, plant species coexist and interact with one another by stimulating or inhibiting growth and development through various molecular interactions (**Bachheti et al., 2020**). These interactions are predominantly based on secondary metabolites, also known as allelochemicals produced and released into the environment by higher plants. Allelochemicals are found in different plant parts, including flowers, leaves, fruits, seeds, rhizomes, pollen, or stems at different levels (**Heig, 2008; Bachheti et al., 2020**). They are often lethal to other species, including insects, weeds, and beneficial and disease-causing micro-organisms. Allelochemicals may be detrimental to the species producing them (**Bachheti et al., 2020**). However, it was reported that allelochemicals play no physiological role in the plants that produce them (**Rice, 1984**). Plants produce hundreds of compounds that are not involved in their primary metabolism (**Duke, 1986**). These chemicals, when released to the surrounding environment through volatilization, decomposition of plant material, root exudation, and leaching from aboveground plant parts, interfere with different plant physiological functions such as seed germination, seedling establishment, plant growth, and development, which subsequently affect susceptible plants (**Rice, 1974**). Allelochemicals have been shown to restrict nutrient uptake or interfere with a naturally occurring symbiotic relationship, thereby depriving plants of valuable sources of nutrients. Allelochemicals are reported to be bio-communicators, which suggest that they are not bioactive as a single compound. However, when they work synergistically with the other biochemicals, they produce remarkable results (**Macias et al., 1998**).

Allelochemicals have been shown to have properties that can attract or repel nematodes. **Rice (1974)** reported that these allelochemicals constitute a diverse group of chemical families classified into 14 groups according to their chemical entities. Bionematicides are bioactive compounds derived from different plant organs and are primarily considered to be secondary metabolites (**Bertinet al., 2003**). Plants use allelochemicals as a defense against diseases, weeds, and parasites (**Cheng and Cheng, 2015**). Allelochemicals are widely used in agriculture and have a sound anti-pesticidal effect (**Rice, 1984; Cheng and Cheng, 2015**). It has been proven that allelochemicals can affect biological systems through density-dependent growth (DDG) patterns (**Salisbury and Ross, 1992**). The DDG patterns consist of three processes: stimulation, consistency, and inhibition (**Salisbury and Ross, 1992**). **Mashela et al**. (**2015; 2017**) reported that the adoption of the stimulation phase to enhance plant growth was attained when using low concentrations. Consequently, at these low levels, nematode population densities were reduced. Several studies investigated the use of allelochemicals in the form of crude plant extracts, leachates, and decomposing residues (**Silva et al., 2014; Haider et al., 2015**; **Mashela et al., 2017**). Possibilities do exist to exploit allelochemicals for nematode control, and there have been many attempts to use this approach as part of the rotation, intercropping, or green manure strategies (**Halbrendt, 1996; Haider et al., 2015; Dtta et al., 2019**). A field study showed that treatment of tomato *(Lycopersicum esculentum)* with *Ageratum conyzoides*, *Azadirachta indica*, *Aegle marmelos*, *Pongamia piñata* and *Brassica campestris* extracts significantly reduced different plant pathogens (**Pattnaiket al., 2012**). Other studies showed that seed germination and seedling growth indices of selected weed species were reduced by increasing wheat extract concentrations, whereas, at lower levels, seed germination was increased (**Ghafarbi et al.,**

2012). **Sithole** (**2016**) observed similar findings when cuttings of *P. sidoides* were exposed to increasing concentrations of *Cucumis myriocarpus* and *Cucumis africanus* extracts.

The adoption of extracts from indigenous plant materials to enhance crop productivity and environmental protection, and as eco-friendly measures to control crop pests and diseases, through bio-agrochemicals derived from allelochemicals, is growing worldwide. An *in vitro* study showed 100% inhibition of egg hatching and larval mortality by concentrated root extracts of siam weed (*Chromolae naodorata*) and neem (*Azadirachta indica*), while castor bean (*Ricinus communis*) and lemongrass (*Cymbo pogon*) exhibited 93 and 95% inhibition of nematode egg hatching and 62.1 and 75% larval mortality, respectively (**Nimbalkar and Rajurkar, 2009**). Similar results were observed by **Kayani et al.** (**2001**) when exposing *Meloidogyne incognita* eggs to root extracts of neem and Dharek (*Melia azedarach*). A 100% inhibition of egg hatching and larval mortality was noted. Studies by **Nimbalkar and Rajurkar** (**2009**) and **Kayani et al.** (**2001**) emphasized that egg inhibition and larval mortality decreased with an increase in the concentration of the extracts.

Furthermore, juvenile mortality increased with an increase in exposure time. These studies are in agreement with observations by **Dube** (**2016**) in which *M. incognita* was exposed to *C. myriocarpus* and *C. africanus* extracts. **Taye et al.** (**2012**) reported a reduction in final nematode population density, root-knot index, and a significant increase in yield per plant and total yields of tomato treated with a 5% concentration of lantana (*Lantana camara*) and Mexican marigold (*Tagetes erecta*) leaf extracts.

2.4.7. Proteomics analysis

The application of bionematicides is considered as a promising, rational, and ecologically safe crop management strategy. Globally, edible and non-edible crops are currently being evaluated for their nematicidal properties. The use of bionematicides is crucial because they are characterized by having high quantities of allelochemicals that can, at certain levels, suppress the population density of PPNs. Furthermore, they are believed to elicit bioactivities via various mechanisms and modes of action against target organisms (e.g. PPNs). Earlier studies have shown that synthetic nematicides affect the central nervous system of most nematodes (**Noling, 1997**). However, PPNs usually develop resistance against synthetic nematicides. Furthermore, they highlighted that a bionematicide could induce inhibitory actions on PPNs at the same level while simultaneously affecting positive physiological changes on the crops, whereas, when PPNs act alone with no inhibitory substances (bionematicides), they can cause severe physiological damages to crops. Studies aimed at understanding the physiochemical changes in plants due to PPN infestations and the proteomic analyses of PPNs using Two-Dimensional Polyacrylamide Gel Electrophoresis (2D-PAGE) have been conducted (**Chatterjee and Sukul, 1981; Molinari et al., 1990; Joubert et al., 2002; Govindasamy et al., 2016**) but are limited and thus further evaluations are required. The Journal of Proteomics and Bioinformatics defined proteomic analysis as "*the complete identification and quantification of the proteome*, *i.e. the complete study of the proteins produced and expressed in the biological systems of an organism at a particular period of time*." **Chatterjee and Sukul** (**1981**) reported on the physiological changes of Ladies' finger (Okra) plants inoculated with *Meloidogyne incognita* and treated with aqueous extracts of *Anthocephalus cadamba*, *Tragia involucrate*, *Peristrophe bicalyculata* and *Aldrin*. The results showed a significant increase in root protein content, with an increase in root galls as

well, when Lady's finger plants were inoculated with *M. incognita* without treatment with extracts. At the same time, extract-treated plants showed a decrease in root galls and root protein content, respectively. Similar results were observed by **Molinari et al.** (**1990**) when the resistance and susceptibility of tomato cultivars were investigated and compared. There is limited information on the impacts of plant extracts on the proteome of plant-parasitic nematodes. Therefore, the study proposes an examination of the effects of bionematicides on the proteome of *Meloidogyne* spp. second-stage juveniles. Findings from such a study may improve the understanding of the interaction between PPNs and plant extracts.

2.5. Overview of medicinal plants used in the current study

Eight different medicinal plant species were selected for testing of nematicidal properties and non-phytotoxicity effects. The selected plants are used mainly to treat various diseases by local people in South Africa and Africa as a continent.

Table 2.3. Information on the selected plant species used in the current study.

Common name	**Scientific name**	**Family**	**Plant part used**	**Voucher specimens**
Pumpkin	*Cucurbita maxima*	Cucurbitaceae	Seeds	-
Bitter almond	*Prunus africana*	Rosaceae	Leaf	NU0090935
Rabassam	*Pelargonium sidoides*	Geraniaceae	Tuberous roots	NU0088178
Forest croton	*Croton sylvaticus*	Euphorbiaceae	Leaf	NU0090934
Bitter apple	*Solanum aculeastrum*	Solanaceae	Leaf	NU0090937

Star-flowered bitter-tea	*Vernonia colorata*	Asteraceae	Leaf	NU0088180
Karee	*Searsia lancea*	Anacardiaceae	Leaf	NU0088179
Blue Squill	*Merwilla plumbea*	Hyacinthaceae	Bulb	NU0090936

Figure 2.3. *Merwilla plumbea* (Lindl.) Speta was previously known as *Scilla natalensis* Planch. (https://species.wikimedia.org/wiki/Merwilla_plumbea).

Merwilla plumbea (Lindl.) Speta, commonly known as blue squill, belongs to the family Hyacinthaceae. Its bulbs are used as medicine in South Africa, mainly in KwaZulu-Natal province (**Sparg et al., 2002**). The warmed bulb is used externally as an ointment for wound healing, treatment of sprains, fractures, boils, and sores, and to draw abscesses. However, the plant is reported to be highly toxic to mammals (**Sparg et al., 2002**). The nematicidal properties of *M. plumbea* are known in nematodes that parasitize humans (**Sparg et al., 2002**). However, their mode of action and degree of phytotoxicity is still largely unknown.

Figure 2.4. *Vernonia colorata* ´ s / de a e -Tree-Vernonia-nthemum-coloratum-subsp-coloratum/).

n m c ctitioners use the aqueous extract of *r a colorata* (Willd.) s e) remedy for the treatment of diabetes (**Sy t al., 2005**), and ethnomedicinal properties for tl m m o i n l orm have been reported (**e 13**). The n t s od of action, and the degree of phytotoxicity are yet be evaluated.

Figure 2.5. *Cucurbita maxima* (Pumpkin) fruits and seeds (https://www.diet-health.info/en/recipes/ingredients/in/dg9392-hokkaido-pumpkin).

Pumpkin (*Cucurbita maxima*) is a cucurbit that is cultivated chiefly for both human and animal consumption. The plant evolved from the European and American continents. However, today it is widely grown in Asia and Africa (**Nishimura et al., 2014**). In South Africa, only the fleshy part of the fruit is consumed, whereas seeds are also consumed in the eastern part of Africa. The seed extracts contain vitamins, linoleic acid, oleic acid, and microelements (**Nishimura et al., 2014**). Seeds of *Cucurbita* spp. are extensively exploited and have been shown to have a significant effect on controlling animal and human nematodes and other ailments, for example, urinal tract disorders (**Salehi et al., 2019a**). However, there is no documented report on its impact on plant-parasitic nematodes.

Figure 2.6. *Croton sylvaticus* (http://pza.sanbi.org/croton-sylvaticus).

Croton sylvaticus is commonly known as "forest fever-berry" and belongs to the family Euphorbiaceae. Its leaves, bark and roots are utilized to treat ailments such as abdominal pains, boils, fever, inflammation, malaria, rheumatism, swellings, and tuberculosis, as well as for ethnoveterinary medicine (**Naidoo, 2008; Maroyi, 2017**). Furthermore, **Maroyi** (**2017**) highlighted the full range of phytochemical properties of *C. sylvaticus,* revealing the presence of alkaloids, flavonoids, lignin, sterols, and tannins. Furthermore, the species exhibits pharmacological activities such as antibacterial, antifungal, anti-inflammatory, antioxidant, larvicidal, and well-documented effects on the central nervous system. However, there is no documented literature stating evidence of the nematicidal properties of this species.

Figure 2.7. *Prunus africana* (https://www.infonet-biovision.org/PlantHealth/MedicinalPlants/Prunus-africana).

Prunus africana belongs to the Rosaceae family, and the species is commonly known as African almond. In 2006, *P. africana* was assessed and listed as vulnerable on the Red List of South African Plants (**Sanbi: http://redlist.sanbi.org/species.php?species=3457-1**). Therefore, this species is under protection in KwaZulu-Natal. The bark is overexploited on a large scale due to its medicinal attributes. In South Africa, the bark is used to treat chest pains, whereas in Europe, the bark extracts have become famous for the treatment of benign prostate hypertrophy. The species is also reported to contain lethal and magical properties (**Nonjinge, 2006**).

Figure 2.8. *Solanum aculeastrum* (http://www.ngkenya.com/flora/solanum_aculeastrum.html).

Solanum aculeastrum belongs to the family Solanaceae and is commonly known as goat bitter-apple. The highly bitter fruit of *S. aculeastrum* is utilized as medicine (fresh, boiled, or burnt) for pain relief in humans and used to treat ringworm in domestic animals (**Welman, 2004**). The fruits, both mature and immature, contain the poisonous alkaloid solanine (**Welman, 2004**). However, there is no documented literature providing evidence of the nematicidal properties of the species.

Figure 2.9. *Searsia lancea* (http://www.plantbook.co.za/searsia-lancea/).

Searsia lancea belongs to the family Anacardiaceae and is commonly known as "*karee*". The fruit is eaten by birds such as bulbuls, guinea fowl, and francolins. *Searsia lancea* leaves provide priceless fodder for livestock (**Madzinga and Kritzinger, 2020**). The tree is also an essential source of shade for livestock in certain regions (**Madzinga and Kritzinger, 2020**). The bark, twigs, and leaves of *S. lancea* contain tannins. The fruits are edible and were once used as an essential ingredient of mead or honey beer (**Madzinga and Kritzinger, 2020**). However, there is no documented literature stating any evidence of *S. lancea* having nematicidal properties.

Figure 2.10. *Pelargonium sidoides* (https://www.agefotostock.com/age/en/Stock-Images/Rights-Managed/SSJ-80081472).

Pelargonium sidoides DC. (African geranium) belongs to the family Geraniaceae, and it is indigenous to the South African region. Its medicinal properties (treatment of upper respiratory tract infections) have been widely exploited by local traditional healers and international pharmaceutical companies since its discovery in 1897 by Charles Henry Stevens, a man who came to South Africa looking for a TB treatment (https://www.acbio.org.za/wp-content/uploads/2015/02/pelargonium-brief.pdf). Today, in South Africa, there are numerous cough remedies derived from *P. sidoides* active ingredients. The abundance of wild *P. sidoides* is in question; the crop is listed on the Red Data List as an endangered species with a possibility of extinction due to over-harvesting, habitat conversion for crop cultivation, and habitat degradation due to livestock overgrazing (**Raimondo et al., 2009**).

Chapter 3: Phytochemical, antioxidant, and mutagenic properties of eight medicinal plants

3.1. Introduction

Plants are considered the number one source of secondary metabolites, with close to a million metabolites produced within the plant kingdom (**Afendi et al., 2012**). Plant secondary metabolites are diverse compounds synthesized from primary metabolites and secreted by plant cells from various metabolic pathways (**Maeda, 2019**). The secondary metabolites produced by plants include phenols, tannins, terpenoids, quassinoids, limonoids, alkaloids, glycosides, and flavonoids (**Cowan, 1999; Chitwood, 2002; Alexan and Ianovici, 2018**), which among other bioactivities have been found to have *in vitro* nematicidal properties (**Chitwood, 2002**). The efficacy of secondary metabolites has been demonstrated in various biological assays, including antiparasitic, antibiotic, antifungal, and antiviral agents (**Cowan, 1999; Savithramma et al., 2011; Thirumurugan et al., 2018; Thawabteh et al., 2019**). Hence, plant secondary metabolites represent essential economic products that can be employed to generate high-value phytochemicals such as drugs, flavours, dyes, fragrances, and insecticides with significant commercial value (**Pagare et al., 2015; Thirumurugan et al., 2018; Tikadar et al., 2020**).

There has been a paradigm shift in plant protection management strategies, with more emphasis now on plant-derived products. Growing environmental concerns associated with chemical controls have accelerated the search and selection for more eco-friendly, safe, and efficient plant-derived pesticides. Thus, the protection of plants against plant-parasitic nematodes using medicinal plant extracts has been well documented (**Pagare et al., 2015; Thirumurugan et al., 2018; Alexan and Ianovici, 2018**). The abundance of secondary phytochemical constituents in

plants is responsible for this uptake. However, this exciting approach is hindered by the potential of induced toxicity. Medicinal plants possess their own natural defense mechanisms, including cytotoxins, genotoxins, and mutagens. These toxins can lead to induced carcinogenicity, endocrine destruction, and different types of endpoint toxicity such as hepatotoxicity, cytotoxicity, ecotoxicity, mutagenicity, and immunotoxicity that can eventually contribute to morbidity and mortality, along with some degree of phytotoxicity (**Atolani and Fabiyi, 2020**). It is also essential to carefully screen the selected medicinal plants for unintended toxic effects before their usage is encouraged. Therefore, plant extracts from *Cucurbita maxima*, *Prunus africana*, *Pelargonium sidoides*, *Croton sylvaticus*, *Solanum aculeastrum*, *Vernonia colorata*, *Merwilla plumbea*, and *Searsia lancea* were prepared in this chapter. These were then utilized to assess the antioxidant activity of methanolic, water, and petroleum ether extracts in a variety of biological tests. Also, qualitative phytochemical screening for alkaloids, flavonoids, phenols, tannins, saponins, steroids, carbohydrates, anthraquinones, and terpenoids was carried out. The total phenolic, flavonoid, and condensed tannin contents were quantified, and the plant extracts' mutagenic properties were determined.

3.2. Material and methods

3.2.1. Plant material collection, location, and preparation of extracts

The test plants, which include *P. africana*, *P. sidoides*, *C. sylvaticus*, *S. aculeastrum*, *V. colorata*, *S. lancea, C. maxima* and *M. plumbea*, were selected based on a reported wide range of pharmacological activities such as anthelmintic, insecticidal, and larvicidal properties, and the use of some as vermicides (**Hutchings et al., 1996; Sparg, 2003; McGaw et al., 2007; Ayaz et al., 2015; Maroyi, 2017; Morah et al., 2019**). All plant species were collected from the

University of KwaZulu-Natal's Botanical Garden in early March 2018. The fruit of *C. maxima* was purchased from a local supermarket, and only the seeds were used in this experiment (**Table 2.2**). Plants were identified by the horticulturist, Mrs. Alison Young, at the Department of Life Sciences, University of KwaZulu-Natal, Pietermaritzburg, South Africa. Voucher specimens were deposited at the UKZN, Herbarium (NU), Pietermaritzburg for botanical verification and future reference (**Table 2.3**).

The collected material was washed thoroughly with sterile, distilled water. Subsequently, clean plant parts were chopped into small pieces and dried in an oven for five days at 50 ºC to minimise the loss of volatile phytochemicals (**Makkar, 1999**). Each dried material was further ground into a fine powder using a grinder (IKA®, USA).

3.2.2. Phytochemical analysis

Before phytochemical assays were carried out, plant extracts were prepared by sonicating 1 g of dried plant material in 20 mL of 50% methanol (v/v) for 20 min. The sample extracts were double-filtered using filter paper (Whatman No. 1). The filtrates were used immediately as a stock solution. Phytochemical screening was carried out on the methanol plant extracts to detect the most important constituents, including alkaloids (Dragendorff's reagent), flavonoids (NH_3 solution), phenols (Braymer's reagent), carbohydrates (Benedict's), tannins (Braymer's reagent), anthraquinones (Borntranger's test), saponins (foam), steroids, and terpenoids (Salkowski) (**Khan et al., 2017**).

3.2.3. Determination of total phenolic content

Total phenolic content in 50% methanol (v/v) extracts of all eight medicinal plant species was determined by the Folin-Ciocalteu assay described by **Makkar** (**1999**) and using gallic acid as a standard. Fifty microlitres of each sample were diluted with 950 µL distilled water, 500 µL 1 N Folin-Ciocalteu phenol reagent (v/v), and 2.5 mL 2% sodium carbonate (w/v) were added sequentially. The assay was done in triplicate. Samples were incubated at room temperature for 40 min, and absorbance was read at 725 nm using a Cary 50 UV–visible spectrophotometer (Varian, Australia). A reaction mixture that contained 50% aqueous methanol (v/v) instead of extracts was used as a blank. Gallic acid was used as standard (10–150 µgmL^{-1}, $Y = 0.002x - 0.002$, $R^2 = 0.979$). The results were expressed in mg of gallic acid equivalents (GAE) per gram of dry extract (mg GAEg^{-1}). All determinations were carried out in triplicate.

3.2.4. Determination of total flavonoids

Total flavonoid content was determined using the aluminum chloride colorimetric assay, according to **Tristantini and Amalia** (**2019**), with slight modifications. Hence, 0.5 mL of each plant extract was mixed with 1.5 mL of distilled water, 0.1 mL of potassium acetate 1 M solution (v/v), 0.1 mL of aluminum chloride 10% (v/v), and 2.8 mL distilled water (v/v). Quercetin solution (10 mgmL^{-1}) was prepared and diluted into concentrations ranging from $10 - 440$ µgmL^{-1} (w/v); thereafter, 0.5 mL of each concentration was added as a substitute for the extract to the reaction mixture. All samples were homogenized using a vortex and incubated for 30 min at room temperature. Subsequently, absorbance at 415 nm was measured using a Cary 50 UV–visible spectrophotometer. A reaction mixture containing 50% aqueous methanol instead of sample extracts was used as a blank. A yellow colouration indicated the presence of flavonoids.

The concentration of flavonoid content in the test samples was calculated from a quercetin calibration plot (Y = 0.003x - 0.079, R^2 = 0.959) and expressed in mg of quercetin equivalents (QE) per gram of extract (mg QEg^{-1}). The assay was carried out in triplicate.

3.2.5. Determination of tannins

Proanthocyanidins were determined using the butanol-HCl assay as described by **Makkar** (**1999**) with slight modifications. Three milliliters of butanol-HCl reagent (95:5 v/v) was added to 0.5 mL of the extract, followed by 0.1 mL of ferric reagent (2% ferric ammonium sulfate in 2 N HCl (v/v)). The reaction mixtures were vortexed and incubated in a boiling water bath for 1 h. After the incubation period, absorbance was recorded at 550 nm using a Cary 50 UV–visible spectrophotometer. Absorbance was measured against a blank that contained the sample extract (500 µL), ferric reagent (100 µL), and butanol–HCl reagent (3 mL), but without heating. The proanthocyanidin content was expressed as mg of cyanidin chloride equivalent (CCE) per g of dry plant material based on a standard curve of cyanidin chloride (50–500 μgmL^{-1}, Y = 0.001x + 0.071, R^2 = 0.979). The assay was performed in triplicate.

3.2.6. Antioxidant activity

2,2-diphenyl-1-picrylhydrazyl free radical scavenging assay

Plant extracts were prepared by soaking 10 g of dried plant material in 100 mL of each of the two solvents (water and methanol) for 24 h on an orbital shaker. The sample extracts were double-filtered using filter paper (Whatman No. 1) and were concentrated to dryness under reduced pressure using a rotary evaporator. In contrast, water samples were reduced to dryness using a lyophilizer, Virtis freezer-drier, BenchTop Pro with Omnitronics^{TM} (United Scientific).

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging ability was evaluated based on the method of **Xie et al.** (**2010**) with some modifications. Butylated hydroxytoluene (BHT) and ascorbic acid (AA) were used as standards in identifying the antioxidant capacity, achievable in terms of DPPH radical scavenging capability of the methanolic extract of the eight medicinal plants. Dried plant extracts were re-dissolved in 50% aqueous methanol (v/v) to a known concentration (1 $mgmL^{-1}$). Thereafter, 10-100 µL of each plant extract was diluted with 50% aqueous methanol (v/v) to make up a volume of 750 µL and then added to a methanolic $DPPH^{\bullet}$ solution (750 µL, 0.1 mM (w/v)). The concentration of $DPPH^{\bullet}$ in the final reaction was 50 µM (**Sharma and Bhat, 2009**). The $DPPH^{\bullet}$ solution was prepared freshly before the assay. The reaction mixtures were prepared under dim light in test tubes, shaken well, and incubated in the dark for 30 min at room temperature. After incubation, the absorbance was recorded at 517 nm against a blank. The assay was performed in triplicate, and the percentage (%) inhibition of free radical species of the plant extracts and synthetic antioxidants was calculated as:

$$\textbf{\% DPPH radical scavenging activity} = \left(\frac{\textbf{absorbanceofcontrol} - \textbf{absorbanceoftestsample}}{\textbf{absorbanceofcontrol}}\right) \times \mathbf{100}$$

The 50% inhibition concentration (IC_{50}) for plant extracts was calculated graphically from a linear regression curve by plotting antioxidant percent (%) inhibition versus the corresponding sample concentration.

3.2.7. Ames test

The Ames Salmonella/microsome assay was used to investigate the mutagenic characteristics of plant extracts using two *Salmonella typhimurium* tester strains, TA1535 and TA102, in the absence of S9 metabolic activation (**Maron and Ames, 1983; Elgorashi et al., 2003**). A cell

density of 1 x 10^9 CFU/mL was achieved by incubating 100 µL of each stock tester strain in 10 mL of Oxoid nutrient broth No. 2 for 16 hours at 37 ºC. In triplicates, 100 µL of 3 dilutions (50, 500, and 5 000 µgmL^{-1}) of each plant extract were mixed with 500 µL of phosphate buffer (0.1 mM, pH 7.4), followed by 100 µL of the tester bacterial strain and 2 mL of sterile melted top agar supplemented with 0.5 mM of biotin and histidine (0.5 mM). The mixture was vortexed, then transferred into minimal agar plates and allowed to solidify for 2-3 min. One hundred microliters of 50% methanol (v/v) served as the solvent control, while 2 µg per plate of 4NQO were used as the positive control. After a 48 h incubation period at 37 ºC, the number of viable colonies in each plate was counted. The assay was repeated twice, with the findings provided as the mean standard error number of reverted colonies per plate. Mutagenic plant samples were defined as those that caused a two-fold increase in the number of His+ revertants as compared to the negative control. Additionally, samples with a dose-dependent increase in the number of His+ revertants were classified mutagenic (**Cariello and Piegorsch, 1996**).

3.2.8. Data analysis

SPSS version 24.0 for Windows® (IBM SPSS Inc., Chicago, IL) was used to analyze the data, and Graph Pad Prism version 5.0 for Windows® (Graphpad Software Inc., San Diego, CA) was used to plot all graphs. The quantification of total phenolic, flavonoids and condensed tannin contents were done in triplicate and the results presented as mean ± standard error. Mean values were compared using one-way analysis of variance (ANOVA). Antioxidant data were analyzed using two-way ANOVA with the statistical significance level set at $p \leq 0.05$. The mean values were further separated using Duncan's multiple range test.

3.4. Results

3.4.1. Phytochemical analyses

Preliminary phytochemical screening of plant extracts of *C. maxima*, *P. africana*, *P. sidoides*, *C. sylvaticus*, *S. aculeastrum*, *V. colorata*, *S. lancea* and *M. plumbea* revealed the presence of alkaloids, terpenoids, phenols, flavonoids, tannins, steroids, saponins, carbohydrates and anthraquinones secondary metabolites (**Table 3.1**). The results demonstrated that total phenolics, flavonoids, and condensed tannin contents varied significantly ($p<0.01$) among the tested plant extracts (**Figure 3.1, 3.2 and 3.3**). Significantly ($p<0.01$), *P. sidoides* and *S. lancea* showed the highest total phenolic contents and condensed tannins (**Figure 3.2 and 3.3**) compared to the other tested crude extracts. On the other hand, high flavonoids were noted in *V. colorata*, followed by *S. lancea* and *C. sylvaticus* crude extracts (**Figure 3.1**). The least flavonoids and total phenolic contents were recorded in *M. plumbea* and *C. maxima*; condensed tannins in these two plant extracts were significantly ($p<0.01$) lower than the rest. Overall, the phytochemical contents of the crude extracts were noted in the following order; total phenolic content: *P. sidoides* > *S. lancea* > *S. aculeastrum* > *V. colorata* > *C. sylvaticus* > *P. africana* > *M. plumbea* > *C. maxima*, total flavonoid content: *V. colorata* > *S. lancea* > *C. sylvaticus* > *S. aculeastrum* > *P. africana* > *C. maxima* > *P. sidoides* > *M. plumbea* and condensed tannin content: *S. lancea* > *P. sidoides* > *P. africana* > *V. colorata* > *C. sylvaticus* > *S. aculeastrum* = *M. plumbea* = *C. maxima* (**Figure 3.1, 3.2 and 3.3**).

Table 3.1. Chemical constituents of methanolic extracts of eight medicinal plants.

Plant extracts	***Cucurbita maxima***	***Prunus africana***	***Pelargonium sidoides***	***Croton sylvaticus***	***Solanum aculeastrum***	***Vernonia colorata***	***Searsia lancea***	***Merwilla plumbea***
Alkaloids	+	-	+	-	+	-	+	+
Terpenoids	+	+	+	+	-	+	+	+
Phenols	+	+	+	+	+	+	+	+
Flavonoids	+	+	+	+	+	+	+	+
Tannins	-	+	+	+	-	+	+	-
Steroids	+	+	+	+	+	+	+	+
Saponins	-	-	-	-	-	-	-	+
Anthraquinones	-	+	-	+	+	+	+	-
Carbohydrates	+	-	+	+	+	+	+	+

Where: - = absent and + = presence

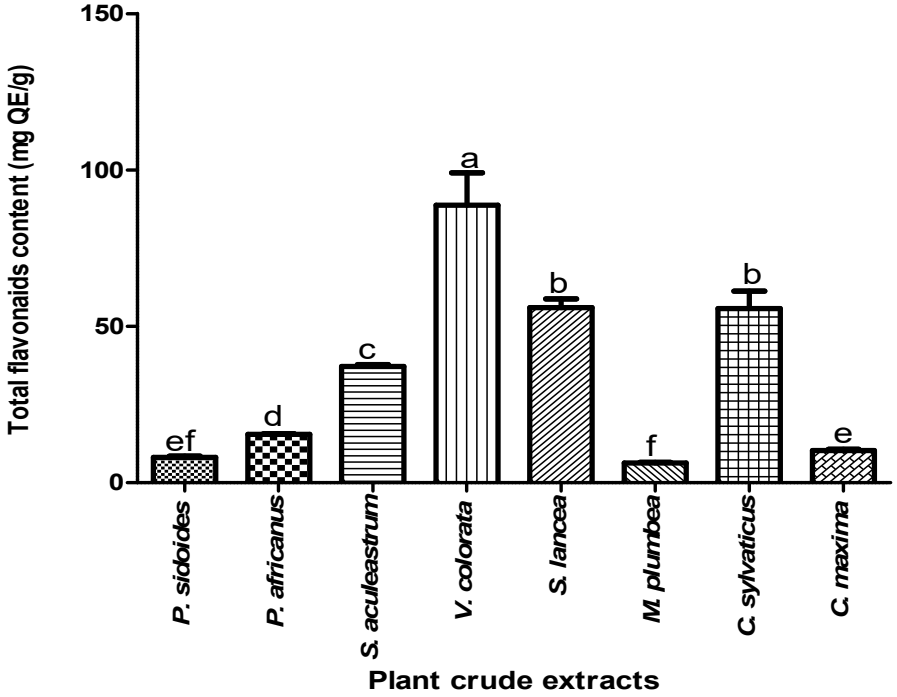

Figure 3.1. Quantity of total flavonoids (QE: Quercetin equivalents) in 50% (v/v) aqueous methanol extracts of eight traditional medicinal plants. The bar represents mean ± standard error, n = 3. According to Duncan's Multiple Range Test, plant crude extracts with different letter(s) are significantly ($p \leq 0.05$) different.

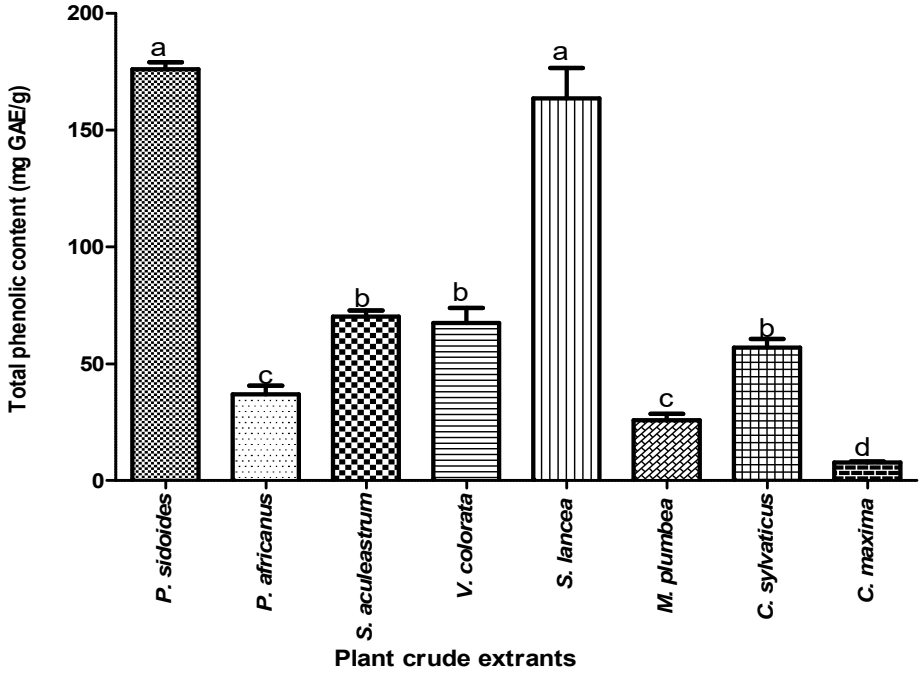

Figure 3.2. Total phenolic content (GAE: Gallic acid equivalents) in 50% (v/v) aqueous methanol plant extracts. Bars represent mean ± standard error, n = 3. Plant crude extracts with a different letter(s) are significantly ($p \leq 0.05$) different as separated by Duncan's Multiple Range Test.

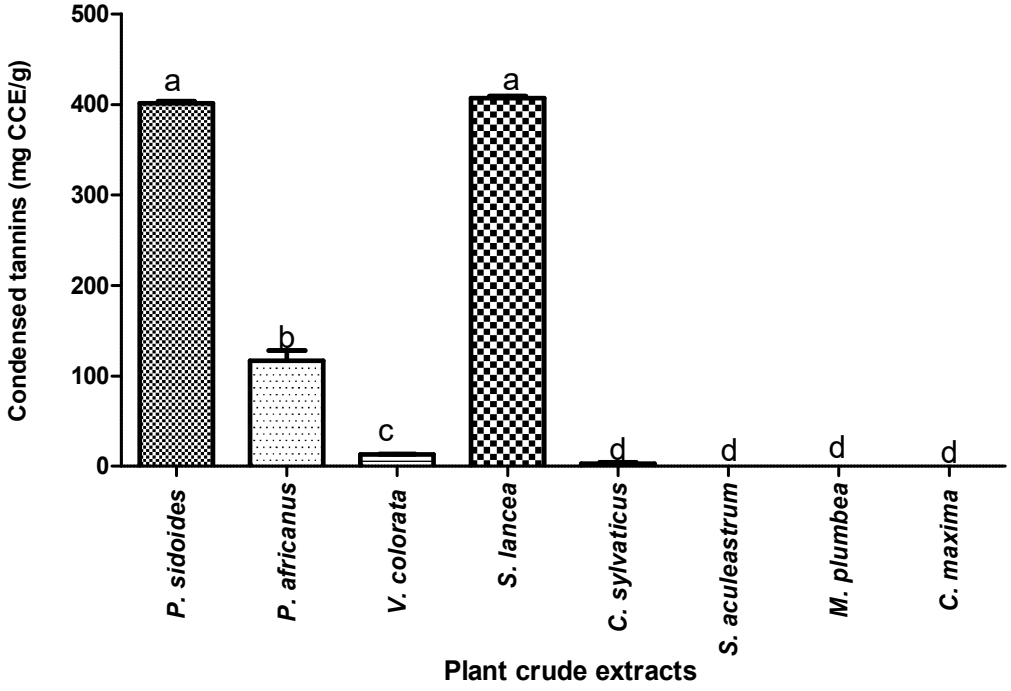

Figure 3.3. Quantity of condensed tannins (CCE: Cyanide chloride equivalents) in 50% (v/v) aqueous methanol extracts of eight plant crude extracts. The bar represents mean ± standard error, n = 3. Plant organs with different letter(s) are significantly ($p \leq 0.05$) different as separated by Duncan's Multiple Range Test.

3.4.2. Antioxidant activity

In comparison to the reference antioxidant standards (butylated hydroxytoluene and ascorbic acid), *C. maxima*, *P. africana*, *P. sidoides*, *C. sylvaticus*, *S. aculeastrum*, *V. colorata*, *S. lancea* and *M. plumbea* showed a significant increase in radical scavenging activity with an increase in extract concentration (**Figure 3.4 to 3.6**). The best scavenging activity and the lowest$IC_{50}$was observed in this order; *P. sidoides* (6.64 μgmL^{-1}) > *S. aculeastrum* (8.11 μgmL^{-1}) > *S. lancea* (14.40 μgmL^{-1}) > *C. sylvaticus* (20.80 μgmL^{-1}) > *P. africana* (39.56 μgmL^{-1}) methanolic extracts

(**Table 3.2**). Furthermore, the water extracts highest scavenging activity and lowest IC_{50} was observed in *S. lancea* (0.84 μgmL^{-1}) > *P. sidoides* (6.74 μgmL^{-1}) > *M. plumbea* (14.40 μgmL^{-1}) > *S. aculeastrum* (17.56 μgmL^{-1}) > *P. africana* (18.43 μgmL^{-1}) > *V. colorata* (38.04 μgmL^{-1}) > *C. sylvaticus* (41.01 μgmL^{-1}) compared to the other medicinal plant species, while, *P. africana, P. sidoides, C. sylvaticus, S. aculeastrum, V. colorata, S. lancea* and *M. plumbea* petroleum ether extract showed good antioxidant activity with IC_{50} values of 26.81, 49.76, 25.69 and 46.53 μgmL^{-1} respectively. Overall, *P. sidoides* and *S. lancea* methanol and water extracts had the best antioxidant activity relative to the reference antioxidant standards (butylated hydroxytoluene and ascorbic acid) with the IC_{50} values of 2.66 and 6.31 μgmL^{-1}, respectively.

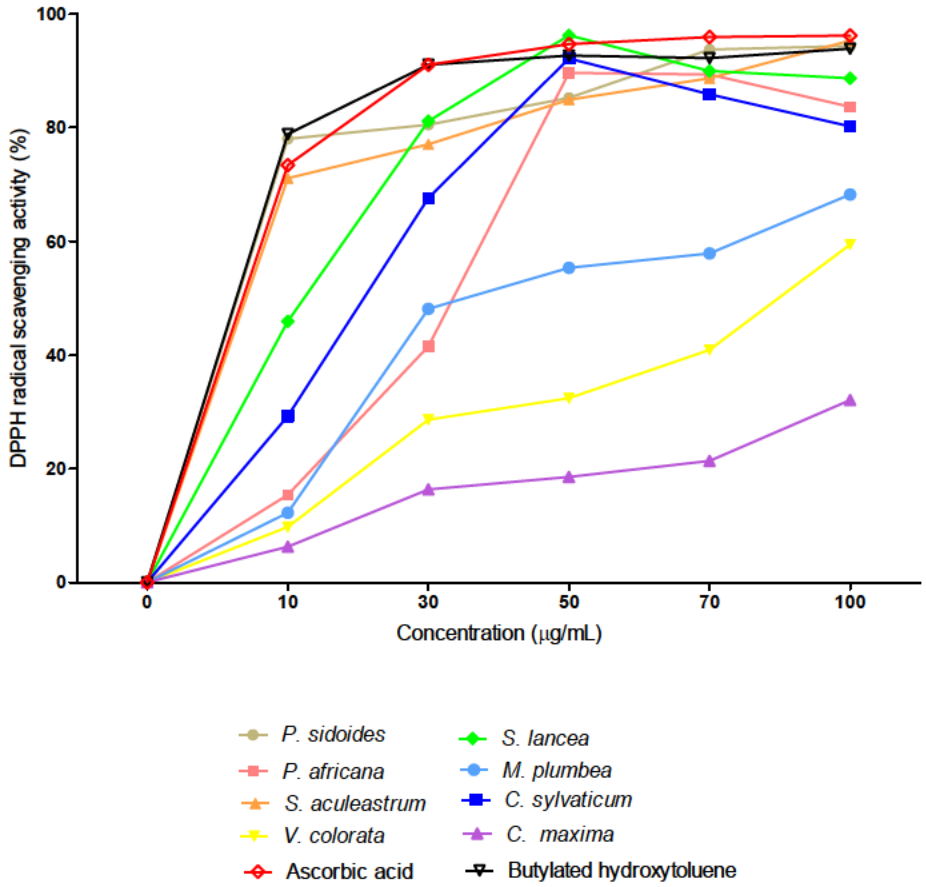

Figure 3.4. Antioxidant activity of the eight medicinal plants' methanolic extracts with butylated hydroxytoluene and ascorbic acid antioxidant activity as positive controls.

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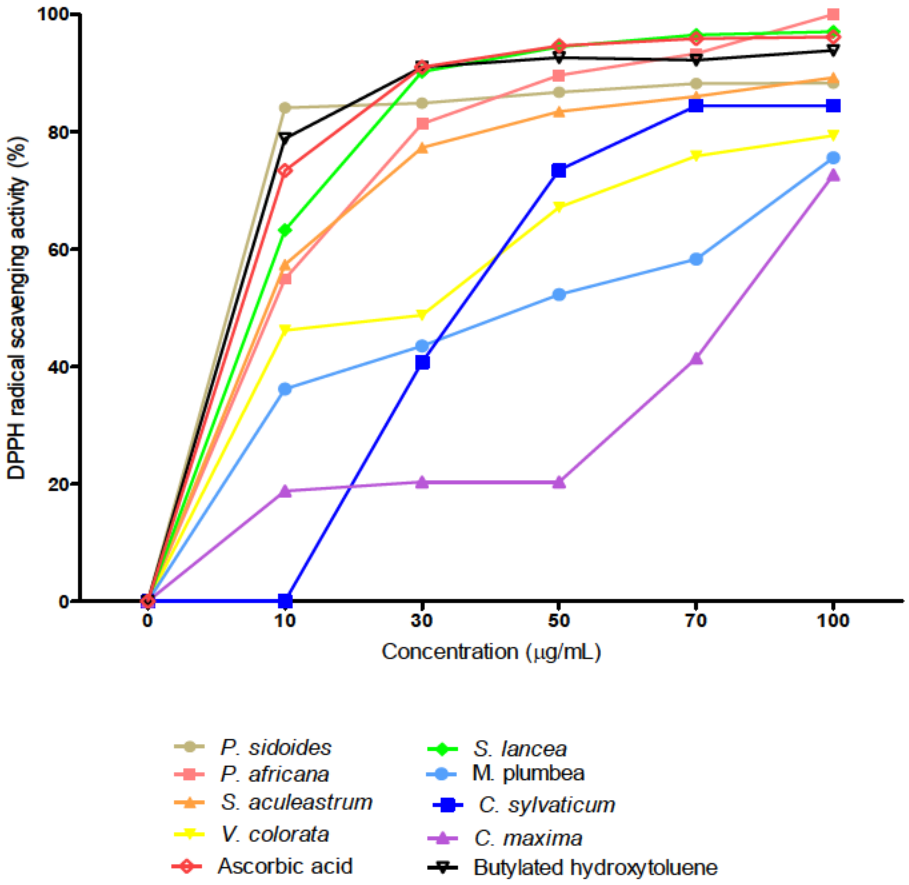

Figure 3.5. Antioxidant activity of the eight medicinal plants' aqueous extracts with butylated hydroxytoluene and ascorbic acid antioxidant activity as positive controls.

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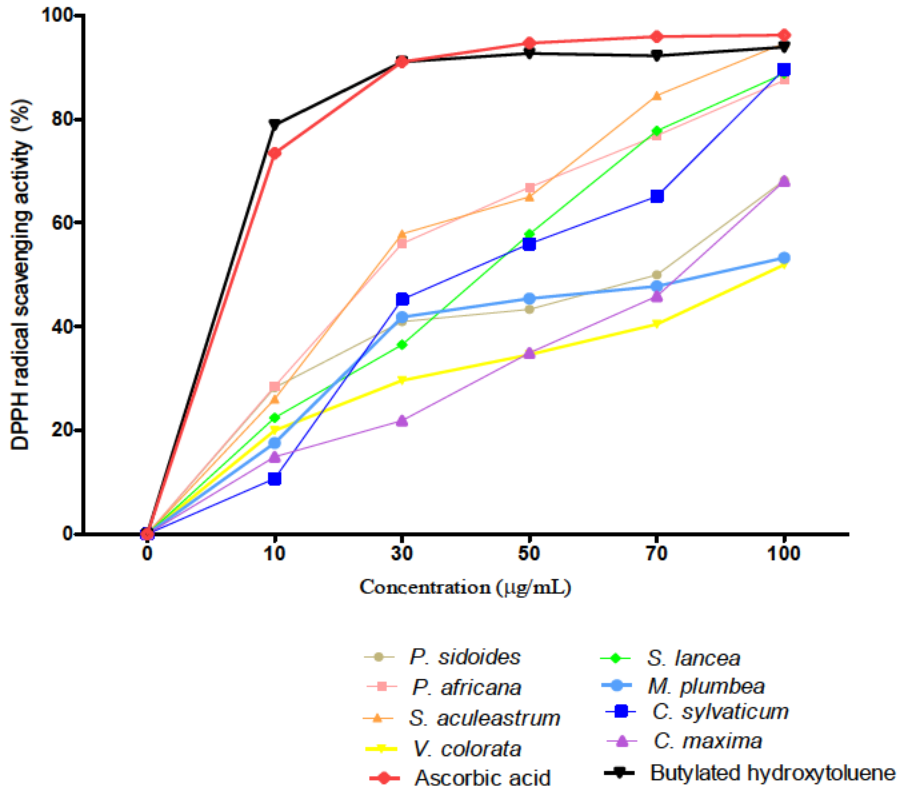

Figure 3.6. Antioxidant activity of the eight medicinal plants' petroleum ether extracts with butylated hydroxytoluene and ascorbic acid antioxidant activity as positive controls.

Table 3.2. DPPH radical scavenging IC_{50} of the eight medical plants.

Treatments	IC_{50} (μgmL^{-1})		
	Methanolic	**Water**	**Petroleum ether**
C. maxima	168.20±16.94	88.51±5.67	67.42±3.56
P. africana	39.56±2.71	18.43±1.23	26.81±2.86
P. sidoides	6.64±1.47	6.74±0.57	62.59±4.10
C. sylvaticus	20.80±1.37	41.01±0.37	49.76±1.27
S. aculeastrum	8.11±1.94	17.56±0.13	25.69±0.87
V. colorata	84.94±3.59	38.04±1.98	89.41±5.08
S. lancea	14.40±0.87	0.84±0.12	46.53±1.23
M. plumbea	51.79±4.71	44.05±2.01	76.26±3.45
Butylated hydroxytoluene	2.66±0.21		
Ascorbic acid	6.31±0.10		

Columns represent mean ±standard error, n = 3.

3.4.3. Ames test

Only methanolic extracts of the test plants were tested for mutagenic effects in the current study due to limited available resources. **Table 3.3.** presents the sum of His^+ collected at three separate concentrations (0.05, 0.5, and 5 $mgmL^{-1}$) for the plant extracts. When a consistent, concentration-dependent increase in the number of revertant colonies in one or more bacterial strains was observed, extracts were considered mutagenic. All the tested plant extracts demonstrated an increase in the number of His^+ revertants with the increased concentration when using the TA102 bacterial strain. In some instances, plant extracts are considered weak mutagens if they generate revertant colonies in a concentration-dependent manner in one or more bacterial strains. Still, the number of revertants was not double the background number of colonies. The current study showed that the average TA102 revertants for all plant extract dilutions ranged from 13.33 to 127.67, while the TA1535 revertants ranged from 1.00 to 6.00. The corresponding

average number of revertants in the positive control (4NQO) were 237.67 (TA102) and 354.00 (TA1535).

Table 3.3. Number of His+ revertants in *Salmonella typhimurium* strains TA1535 and TA102 produced by the selected methanolic plant extracts (without S9 metabolic activation).

		Concentrations ($mgmL^{-1}$)		
Plant species	**Strains**	**5**	**0.5**	**0.05**
P. sidoides	TA102	99.00±6.66	88.00±7.23	35.00±9.45
	TA1535	6.00±1.53	5.33±2.73	1.00±0.58
P. africana	TA102	111.33±10.48	91.00±5.51	79.00±16.17
	TA1535	5.33±2.03	3.00±0.58	4.33±1.45
S. aculeastrum	TA102	71.33±10.20	60.67±8.37	37.33±2.33
	TA1535	5.33±0.33	2,67±0.33	5.00±1.15
V. colorata	TA102	108.67±5.78	77.00±1.53	74.33±24.52
	TA1535	3.00±1.00	3.00±1.15	2.67±1.20
S. lancea	TA102	93.67±4.91	36.67±5.78	34.00±8.02
	TA1535	4.67±2.03	4.33±0.33	3.67±0.88
M. plumbea	TA102	127.67±27.68	87.67±3.53	51.00±18.01
	TA1535	4.33±1.20	4.00±0.58	5.33±0.33
C. sylvaticus	TA102	93.67±9.82	77.33±7.51	65.67±10.39
	TA1535	4.33±0.67	5.67±2.33	5.33±1.86
C. maxima	TA102	80.00±4.07	33.33±8.01	13.33±2.19
	TA1535	4.33±0.67	5.33±0.88	3.00±1.53
4NQO (+ve control)	TA102		237.67±25.77	
	TA1535		354.00 ± 9.54	
Water (-ve control)	TA102		42.66±11.69	
	TA1535		3.67±0.33	

Columns represent mean ± standard error, n = 3.

3.4. Discussion

3.4.1. Profiling of phytochemical and antioxidant properties

The current study investigated the phytochemical contents and antioxidant properties of *C. maxima, P. africana, P. sidoides, C. sylvaticus, S. aculeastrum, V. colorata, S. lancea* and *M. plumbea* crude extracts. The tuberous roots of *P. sidoides* contained all the phytochemicals that were screened for, except saponins. Previous reports by **Kolodziej** (**2007**) and **Moyo and Van Staden** (**2014**) noted the presence of phenolics, flavonoids, and proanthocyanidins, while **Kolodziej and Schulz** (**2003**) reported good antioxidant activity in *P. sidoides*, which agrees with the current findings. Similar antioxidant results were reported by **Kumar et al.** (**2015**).

The stem bark of *P. africana* is well studied due to its medicinal properties. Traditionally the bark is chewed, or the ground material is orally administered like tea to manage various infections (**Stewart, 2003**). The current study showed the presence of terpenoids, phenols, flavonoids, tannins, steroids, and anthraquinones in *P. africana*. **Ngeranwa et al.** (**2020**) previously reported the presence of all these compounds, except anthraquinones, in addition to others like carbohydrates, saponins, alkaloids, quinines, cardiac glycosides, phenols, steroids, and coumarin in the stem bark of *P. africana*. Although the present study only evaluated the phytochemicals and antioxidant activity of *P. africana* leaf extracts, other parts of *P. africana* consist of phytochemicals considered significant in ethnomedicine.

On the other hand, *Solanum aculeastrum* is a medicinal plant from subtropical Africa. Traditionally, *S. aculeastrum* fruit is used to treat various diseases in humans and domestic animals. The current study tested the phytochemical and antioxidant activity of *S. aculeastrum*

leaves. The most significant phytochemicals noted from this plant were alkaloids, phenols, flavonoids, steroids, anthraquinones, and carbohydrates. The leaves of *S. aculeastrum* also showed good antioxidant activity. Similar observations were made by **Koduru et al.** (**2007**) from their analysis of *S. aculeastrum* berries.

Croton sylvaticus contains a wide range of phytochemicals and has pharmacological activities; it has been shown to treat about 24 human and animal ailments. The presence of phytochemicals such as anthraquinones, alkaloids, flavonoids, lignin, phenolics, sterols, tannins, terpenoids, and essential oil from leaves, roots, and stems have been reported (**Maroyi, 2017**). In this study, we observed the presence of anthraquinones, flavonoids, phenolics, steroids, tannins, terpenoids, and carbohydrates in *C. sylvaticus* leaf extracts. The aqueous, methanol and petroleum ether extracts displayed DPPH antioxidant activity with an IC_{50} of 41.01 ± 0.37 μgmL^{-1}, 20.80 ± 1.37 μgmL^{-1}, and 49.79 ± 1.27 μgmL^{-1}, respectively. Similarly, phytochemical screening of *Vernonia colorata* leaf extracts showed anthraquinones, flavonoids, phenolics, steroids, tannins, terpenoids, and carbohydrates. The DPPH potential of *V. colorata* leaf extracts prepared using methanol and petroleum ether as solvents were rather low. In contrast, the aqueous, methanol and petroleum ether extracts showed good activity with the increasing concentration with IC_{50} of 84.94 ± 3.59, and 89.41 ± 5.08 and 38.04 ± 1.98 μgmL^{-1}, respectively. **Guenne et al.** (**2012**) reported similar qualitative phytochemical results, with lower total flavonoid and tannin contents and higher total phenolic contents. However, a literature search on the phytochemical and antioxidant evaluation of these species generated little information. The current study revealed that all screened phytochemicals were present in *S. lancea* leaf extracts, except for saponins. These species showed high levels of total phenolic, flavonoids, and condensed tannins, similar to the results

reported by **Vambe et al.** (**2018**). The DPPH radical scavenging activity of the crude extracts is concentration-dependent irrespective of the extraction solvent. *C. maxima* crude extract showed the lowest radical scavenging activity, with aqueous seed extracts demonstrating high activity at concentrations of 100 μgmL^{-1}. This agrees with recent findings by **Muchirah et al.** (**2018**) which documented good *C. maxima* antioxidant activity at 10 $mgmL^{-1}$. **Annapandian and Rajagopal** (**2017**) explained that the higher $DPPH^{\bullet}$ scavenging activity could be attributed to the extracts' proton-donating ability, which tends to stabilize the free radicals in association with several hydroxyl groups. Hence, in many cases, the total phenolic compounds and other antioxidants form a positive relationship due to the hydroxyl group's presence, which has a scavenging ability (**Zhao et al., 2014**). Therefore, our results align with the earlier reports by **Zhao et al.** (**2014**), where a positive correlation between total phenolic contents and antioxidant activity was shown.

In the current study, a decrease in DPPH radical scavenging activity was noted at the highest concentrations of *C. sylvaticus* and *P. africana* crude methanolic extracts. Similar findings were reported by **Nguyen et al.** (**2013**) and **Abifarin et al.** (**2019**), which was due to the scavenging capability of soluble solids within the concentration of crude extracts they tested. Methanolic, water, and acetone crude extracts of *C. africanus* showed good DPPH radical scavenging activity (**Abifarin et al., 2019**). In contrast, those with low total phenols had a more moderate antioxidant activity. Extracts of *P. sidoides* and *S. aculeastrum* exhibited a potential to scavenge DPPH radicals similar to BHT and AA, while *C. maxima* extracts showed the least activity. Except for *P. sidoides* water extract, all extracts tested showed radical scavenging activity in a dose-dependent manner. These agree with earlier findings by **Motalleb et al.** (**2005**). They noted that the DPPH radical scavenging effects increased steadily with an increase in the extracts and

standards' concentration. This study showed that the maximum absorbance for methanol extracts was achieved at 50 µgmL^{-1} for *P. africana*, *S. lancea* and *C. sylvaticus* extracts, respectively. While water extracts yielded maximum activity at 100 µgmL^{-1}, *P. sidoides*, *S. lancea* and *C. sylvaticus* extracts showed maximum activity at concentrations less than 100 µgmL^{-1}. Due to high flavonoid and phenolic contents, *P. sidoides* extracts showed relatively good DPPH radical scavenging activity. At the highest concentration of 100 µgmL^{-1}, the radical scavenging activity of methanol extracts decreased in the following manner for *P. africana* > *S. lancea* > *S. aculeastrum* > *P. sidoides* > *C. sylvaticus* > *M. plumbea* > *V. colorata* > *C. maxima*; while water extracts showed the following decreasing order of scavenging activity, *S. aculeastrum* >*P. sidoides* > *S. lancea* > *P. africana* > *C. sylvaticus* > *V. colorata* > *M. plumbea* > *C. maxima.* This decreasing order of antioxidant activity revealed that a smaller quantity of the extract having higher radical activity is needed to inhibit 50% of DPPH free radical. In contrast, a more significant amount is required for extracts that showed low activity. This study demonstrates the plants' medicinal significance; it is enlightening that all the plant extracts were active species for antioxidant activity due to phenolics and flavonoids, which can stabilize the free radicals into neutral compounds.

3.4.2. Effect of secondary metabolic on plant-parasitic nematodes

Bioactive compounds from several plants have been reported to play a vital role in managing pests and diseases (**Mwanauta et al., 2014**). These bioactive compounds are collectively known as phytonematicides due to the synergistic effects of the active allelochemicals, known as phytoconstituents, such as flavonoids, phenols, alkaloids, steroids, saponins, tannins, glycosides, terpenoids, etc. (**Chitwood, 2002**). **Trifonova and Atanasov** (**2009**) reported that some

phytochemicals of plant extracts show lipophytic properties, a property that allows the cytoplasmic membrane of nematodes to dissolve. This interferes with their enzyme and protein structure. Generally, different phytochemicals' nematicidal activities have been previously reported (**Chitwood, 2002; Batish et al., 2008; Chin et al., 2018; El-Deriny et al., 2020**). The key mode of action for these phytochemicals includes their contribution to the chemotactic, repellent, or attraction of nematodes (**Warnock et al., 2016; Chin et al., 2018**). **Chitwood** (**2002**) illustrated that phytochemicals present in some plant extracts consequently had a significant inhibitory effect on egg hatching fecundity in *Meloidogyne* spp. **Warnock et al.** (**2016**) reported that monosaccharides such as fructose and glucose attract and activate larvae development in *M. incognita.* **Wuyts et al.** (**2006**) reported the nematicidal effects of phenylpropanoids (simple phenolics and flavonoids) and selected monoterpenoids and alkaloids against migratory and sedentary endoparasites *Radopholus similis* and *Pratylennchus penetrans*, and *M. incognita*, respectively. **Wuyts et al.** (**2006**) further detailed that phloretin displayed inhibitive properties against *P. penetrans* egg hatching. In contrast, simple phenolic compounds were noted to act as repellents and motility inhibitory activities when tested against *R. similies* and *M. incognita*, with flavonol compounds exhibiting exceptional repellent activity against both nematodes species. Also, flavonol compounds in their degradable form showed inhibitory properties of motility for *M. incognita*. Overall, **Wuyts et al.** (**2006**) stated that larval mortality and retarded egg hatching of *M. incognita* were demonstrated by flavonoids and phenolics. According to **Wuyts et al.** (**2006**), certain flavonoids repel plant-parasitic nematodes, but the effect appears to be more species-dependent for these compounds. For example, the flavonoids such as kaempferol, quercetin, and myricetin repelled *R. similis* and *M. incognita*, but not *P. penetrans* and other flavonoids, such as luteolin, daidzein, and genistein only showed repellence

properties against *R. similis*. **Bird** (**1959**), **Hewlett et al.** (**1997**) and **Maistrello et al.** (**2010**) have reported that tannins have repellents, attractants, and sometimes, no observable effects against some phytonematodes. While **D'Addabbo et al.** (**2011**) reported that the impact of pure saponins and ground biomass of *Medicago sativa* against plant-parasitic nematodes were comparable to that of the inorganic nematicide fenamiphos. It has been reported that anthraquinones have nematicidal effects against gastrointestinal nematodes (**Midiwo et al., 2002**), while **Tripathi et al.** (**2014**) reported its nematicidal effect against *M. incognita*. The impacts of plant extracts on egg hatching and juvenile mortality are directly influenced by the concentration used and the plant species. In most cases, the nematode population has been reduced at a very low concentration, whereas the opposite can still occur depending on the plant species (**Trifonova and Atanasov, 2009**). The family of Asteraceae contains plants that confer nematicidal effects due to the presence of thiarubrine C, a compound with broad-spectrum toxicity that requires light for maximum activity (**Chitwood, 2002**). When thiarubrine C was applied as a soil treatment, it decreased *M. incognita* infection of tomato seedlings by almost 95% at 50 μgmL^{-1}. Plants such as *Pelargonium graveolens* (Geraniaceae), *Cucumis sativus* (Cucurbitaceae), and *Rhus aromatic* (Anacardiaceae) have been shown to contain nematode-antagonizing compounds (**Chitwood, 2002**). The *Cucumis myriocarpus* fruit contains tetracyclic triterpenoids (cucurbitacin B) (**Chen et al., 2005**), alkaloids, phenolics, flavonoids, proanthocyanidin, and saponins (**Abifarin et al., 2019**) with nematicidal properties (**Dube and Mashela, 2016**).

3.4.3. Toxicity or mutagenicity of the screened medicinal plants

All the tested plant extracts were non-mutagenic against the Salmonella tester strains TA1535 and TA102 based on the current study results (**Cariello and Piegorsch, 1996**). However, when tested against TA102, all plant extracts showed a possibility of being weak mutagens. The cytotoxicity and mutagenic evaluations of *C. sylvaticus* extracts by **Kapingu et al.** (**2012**) and **Aderogba et al.** (**2013**) demonstrated that *C. sylvaticus* may be toxic or contain some cytotoxic compounds. According to **Chukwujekwu et al.** (**2009**), when using the MTT-assay, isolated compounds vernodalin and vernolide from *V. colorata* showed potential cytotoxic effects. Extract of *P. sidoides* exhibited cytotoxicity against human lung cancer GLC4 and cell lines of human colon cancer COLO320 (**Kong et al., 2009**).

3.5. Conclusions

Globally, there are numerous different strategies to control soil-borne diseases. The most broad-spectrum approach that most farmers depend on is chemical control, followed by other methods such as biological control, trap crops, resistance varieties, crop rotation, cover crops, cultural controls, and lastly, the employment of soil solarization. However, these management strategies are time-consuming and expensive to undertake. On the other hand, environmental contaminations with chemical pesticides have become a significant problem in the pest control system. Chemical nematicides are the most widely utilized strategy against plant-parasitic nematodes, and at the same time, form part of environmental pollutants. Therefore, it is of importance to search for alternatives that (a) consist of novel compounds that PPNs are not yet able to resist, (b) are less concentrated and toxic to the environment, (c) are biodegradable and (d) are from renewable material (**Chitwood, 2002; Ferraz and Grassi de Freitas, 2004**). Plant-

derived bionematicides uphold a significant role in PPNs controls due to various phytochemicals that have become a promising tool to use as nematicides. To date, studies have revealed that secondary metabolites of plants possess nematicidal properties. Therefore, this Chapter has revealed the presence of phytochemicals and antioxidant activity of *C. maxima, P. africana*, *P. sidoides*, *C. sylvaticus, S. aculeastrum*, *V. colorata, M. plumbea* and *S. lancea* with a promising effective potential to be utilized as phytonematicides. Hence, it was necessary to further evaluate these plants for nematicidal activity under *in vitro* and greenhouse conditions. This set of eight medicinal plants has not been reported for their nematicidal properties against plant-parasitic nematodes. The methanolic crude extracts exhibited a concentration-dependant increase in the revertant colonies in TA102 bacterial strain without S9. In contrast, TA1535 showed no signs of mutagenicity in all tested plant extracts. However, to conclusively declare a plant toxic or mutagenic, other complementary assays and test systems need to be examined since this plant material will be applied to protect consumable crops.

CHAPTER 4: Effects of the eight medicinal plants on *Meloidogyne incognita* and rhizospheric bacteria activity under *in vitro* conditions

4.1. Introduction

Plant-parasitic nematodes (PPNs) affect various plants, thereby intensifying the difficulty to manage the threat (**Khalil and Darwesh, 2008; Kokalis-Burelle and Rosskopf, 2012; Khalil, 2013a; Bernard et al., 2017**). Worldwide, PPNs are cited as the second most important cause of crop losses following pests. Globally, crop losses resulting from PPNs are estimated at 12% annually (**Nyaku et al., 2017**). Root-knot nematodes (*Meloidogyne* species) are the most destructive PPNs. They infest more than 2000 host plants, including ornamentals, weeds, medicinal plants, and edible crops (**Khalil, 2013a; Bernard et al., 2017**). Recent reports show losses in several crops such as tomato, soybean, grapes, citrus, potato, beetroot, peanuts, spinach, dry bean, lettuce, eggplant, maize, and carrot in different parts of the world (**Onkendi et al., 2014; Visagie et al., 2018; Rashidifard et al., 2018; Rashidifard et al., 2019**). The management of PPNs has been reliant on the application of synthetic nematicides. However, alternative strategies aimed at managing nematodes have necessitated the withdrawal of synthetic nematicides from the agrochemical markets. This was due to synthetic nematicides' hazardous consequences to human and environmental health (**Jallow et al., 2017**). Also, chemical measures are rarely available and are too expensive for many growers to purchase (**Jallow et al., 2017**). Phasing out of methyl bromide globally has coincided with increased crop losses estimated at US$125 billion per annum (**Chitwood, 2003**). Several alternative strategies that are relatively inexpensive, effective, and environmentally-friendly (**Asif et al., 2016**) have been proposed as substitutes to synthetic nematicides to manage PPNs. Studies in the last few decades show that thousands of plant species with insecticidal properties have been

identified (**Niroumand et al., 2016; Gakuubi et al., 2016**). On the other hand, both poisonous and non-poisonous plant species, from 57 families, are known to possess nematicidal activity (**Andrés et al., 2012**). This study was conducted to evaluate eight medicinal plants' nematicidal properties on egg hatching and juvenile mobility activity and antibacterial activity against rhizospheric bacteria using the serial dilution method.

4.2. Material and methods

4.2.1. Collection and preparation of plant material

Medicinal plant parts were collected and ground into fine powders as described previously (**Chapter 3; Section 3.2.1**). Ten grams of powder was soaked in 100 mL of absolute methanol and water respectively for 24 h on an orbital shaker (**Daniel and Mammen, 2016**). The extracts were double-filtered using Whatman No. 1 filter paper and concentrated to dryness under reduced pressure using a rotary evaporator. The dried extracts were stored in a refrigerator. Before use, 1 mg was re-dissolved in 1 mL 0.5% aqueous methanol (v/v) and water, respectively.

4.2.2. Egg hatching bioassay

Pure cultures of *M. incognita* were maintained in a greenhouse-grown susceptible tomato cv. Roma V.F. plants, at the University of KwaZulu-Natal, Botanical Garden, before using, the nematode species were identified by Dr. Z.P. Dube from the University of Mpumalanga, Mbombela Campus, South Africa. The effect of five different concentrations of extracts from the eight selected medicinal plants was evaluated for their suppression on egg hatching of *M. incognita* during an *in vitro* experiment. The extracted crude plant samples were reconstituted in distilled water and 0.5% aqueous methanol (v/v). Then parallel

trials for each plant extract were pipetted into a 96-well plate. Roots of nematode-infested tomato were collected from the cultures and washed in running tap water to remove soil particles. After that, the roots were placed on a tray, and a single dark brown egg mass (±300 eggs) was collected using a clean toothpick, dipped in 0.5% sodium hypochlorite (NaOCl) for a few seconds, and placed in a single well of a 96-well plate. The experimental layout was in a completely randomized design with four concentrations of 0.2, 0.4, 0.6, 0.8 mg/mL of plant extracts, and distilled water serving as a control. Each concentration was replicated three times and incubated in an incubator at 25 ± 3 °C. Hatched second-stage juveniles (J2s) were counted under a stereomicroscope after incubation periods of 24, 48 and 72 h. The experiments were repeated twice for each treatment.

4.2.3. The effect of plant extracts on hatched J2s penetration under greenhouse conditions

To verify nematode recovery from the effects of crude methanolic plant extracts, the population of nematodes that ingressed 30-day-old tomato seedlings after inoculation was recorded. The study was conducted under greenhouse conditions at the University of KwaZulu-Natal Botanical Garden (290 37.55′ S; 300 24.13′ E), Pietermaritzburg Campus. The day/night temperatures averaged 28/21 ºC, and maximum temperatures were controlled using thermostatically-activated fans. Thirty plastic pots (6 cm in diameter) were arranged at 0.3 m inter-row and 0.25 m intra-row spacing and filled with 0.2 L steam-autoclaved acid-washed sand. Four-week-old tomato seedlings (cv. Roma V.F.) were transplanted into the pots. Five matured egg masses from each treatment in the *in vitro* study at 0.8 mg/mL concentration were inoculated. Irrigation was conducted every other day while fertigation with 2.5 g per 1 L of Shiman Prescription Mix 2:1:2 (45) + Trace elements (TE: boron, magnesium, zinc, molybdenum, iron, manganese, and copper)

was applied weekly. At thirty days post inoculation, the following data were collected; root galls were assessed using the North Carolina Differential Rating Scale of 0 = no galls, 1 = 1-2 galls, 2 = 3-10 galls, 3 = 11-30 galls, 4 = 31-100 galls, and 5 = >100 galls (**Taylor and Sasser, 1978**). Thereafter, tomato roots were stained with acid fuchsin as described by **Byrd et al. (1983**) (**Figure 4.1**). Briefly, the number of J2s was counted using a stereomicroscope to verify the density of J2s that invaded the roots (**Figure 4.2**). The roots were washed under running tap water and immersed immediately in a 150 mL beaker containing 1 % NaOCl (w/v) for 4 min under agitation. Afterwards, the roots were recovered and rinsed under running tap water for 45 seconds, and to further remove the reminance of disinfectant, the roots were soaked again for 15 min in a beaker containing tap water. Thereafter, the water was drained and the roots were transferred into a new beaker containing 30 mL of tap water followed by adding 1 mL of stock acid fuchsin stain solution (3.5 g acid fuchsin in 250 mL acetic acid and 730 mL distilled water) and allowed to boil on a hot plate for 2 min. The mixture was allowed to cool to room temperature, thereafter the solution was drained and roots were rinsed with tap water. To destain the previously stained roots, 10 mL of glycerine acidified with a few drops of 5N HCl was heated to 95 ºC on a hot plate, and the stained roots were placed in the solution for a few seconds. On cooling, roots were placed on Petri dishes and a few drops of acidified glycerine were applied to the surface of the roots. J2s were counted using a stereomicroscope with the aid of the contrast between root tissue and nematodes.

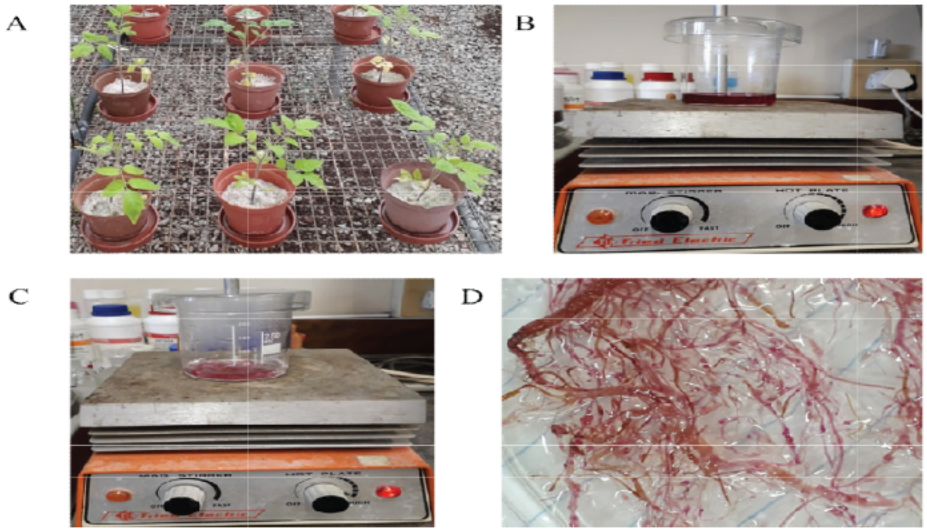

Figure 4.1. c for roots of the tomato plants. A = o s inoculated with *M. incognita*. B = Stai ots after harvest. C = Destaining of roots. D = Stained root galls after destaining.

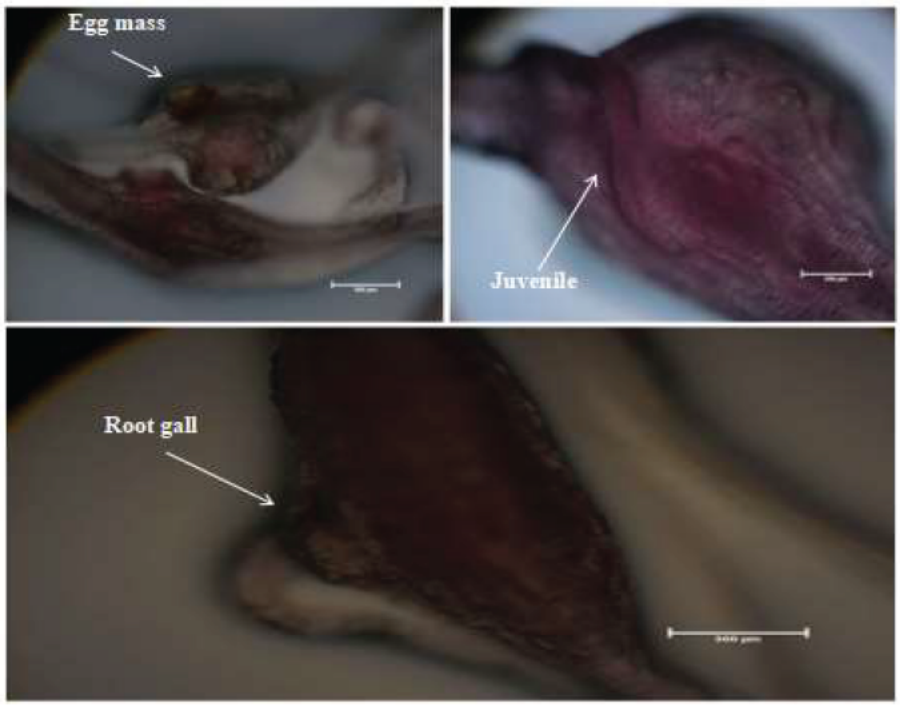

Figure 4.2. Visual of egg mass, j l n s o o s t 30 days of gg e u er microscope.

4.2.4. Juvinile mobility and mortality bioassay

In vitro trials were conducted in the location described previously (**Section 4.2.3**). Crude extracts of the selected medicinal plants were prepared as explained previously (**Section 4.2.1**). Egg masses of *M. incognita* were obtained from two-month-old tomato plants. The egg masses were then placed in Petri dishes containing distilled water in an incubator set at 25 ± 2 ºC. Juveniles that hatched in the first 24 h were discarded, and those that hatched in the subsequent 48h were used in the bioassay. Mobility bioassay of the crude extract concentrations were tested for their inhibition of nematode motility using a modified method of **Wuyts et al.** (**2006**) in eight parallel trials for each solvent. The assessment was carried out using crude extracts in 9-cm-diameter Petri dishes containing 10 mL of 5, 2.5, 1.25, 0.625, 0.313, 0.156 $mgmL^{-1}$ replicated three times. Distilled water and methanol (0.5% v/v) were used as control. Approximately 100 freshly hatched J2s were added to each concentration. In all trials, treatments were replicated three times and arranged in a completely randomized block design in an incubator at 25 ± 2 ºC for 24, 48 and 72 h. After the pre-allotted time intervals, each dish was emptied into a counting chamber, and the mobile and immobile nematodes were counted under a stereomicroscope. Nematodes were considered immobile when no movement was observed for two seconds, even after mechanical prodding with a bristle. Concentration was considered inhibitive of nematode motility when significantly more nematodes became immobilized compared to the control. After 72 h, a possible reversal of juvenile immobility was assessed under a stereomicroscope as described by **Javed et al.** (**2007**), with minor modifications. Juveniles were carefully removed from each treatment using a pipette onto a new Petri dish and diluted with 5 mL of distilled water. The J2s mortality bioassay was conducted similar to the mobility bioassay. However, only plant extracts that showed J2s immobility at 5 $mgmL^{-1}$ were tested for mortality activity. After

72 h, nematodes were stained in 0.015% methylene blue (w/v) for 1 h. All dark blue stained nematodes were considered dead (**Saifullah, 2002**). In all trials, three independent experiments were conducted.

4.2.5. Plant-growth promoting rhizobacteria (PGPR) sample collection and inoculum preparation

Tomato plants were grown for one month in soil collected from the Botanical Garden, and the root samples were harvested then shaken by hand to remove the soil. Rhizospheric bacteria were isolated from 1 g of roots by serial-dilution plating (**Khan et al., 2018**) on nutrient agar (NA; Oxoid Chemicals, Loughborough, United Kingdom). Inoculated Petri dishes were incubated at 25±2 ºC. After 48 h, nine isolates with different morphological appearances on NA were selected. Individual colonies were picked using a nichrome wire loop and streaked onto new plates to obtain pure cultures. Before use, pure cultures of bacteria isolates were maintained on NA slants and stored at 4 ºC. *Bacillus licheniformis* (ATCC 12759) and *Pseudomonas fluorescens* (ATCC 13525) were purchased from the American Type Culture Collection (ATCC). Strains were revived from storage at −70 ºC by streaking a single Micro bank bead onto the fresh nutrient agar (NA) and incubating the plates at 25±2 ºC in an incubator.

4.2.6. Antibacterial activity

Ten grams of each ground plant material was soaked in 100 mL of absolute methanol for 24 h on an orbital shaker and was double-filtered using filter-paper (Whatman No. 1). The plant extracts were concentrated to dryness under reduced pressure using a rotary evaporator (Buchi 461, Minhang District, Shanghai, China). Antimicrobial activity was evaluated using the micro-

dilution assay (**Eloff, 1998**) as described by **Aremu et al.** (**2010**) with minor modification. Briefly, the extracts were re-dissolved in 10% dimethyl sulfoxide (DMSO). The test microorganisms (*B. licheniformis*, *P. fluorescens,* and the nine unidentified rhizobacteria isolated from tomato roots) were cultured overnight at 37 ºC in Mueller-Hinton (MH) broth. They were diluted with sterile MH broth to achieve a final inoculum of approximately 1×10^6 CFU/mL. One-hundred microlitres of each re-dissolved extract was serially diluted (two-fold) with 100 µL sterile distilled water down the wells in a 96-well microtitre plate for each bacterium. Neomycin (100 μgmL^{-1}) (Sigma-Aldrich, Germany) was used as the positive control, whereas broth, water, and 10% DMSO were included as negative controls against the tested bacterial strains. The inoculated microtitre plates were covered with parafilm and incubated at 37 ºC for 24 h. After incubation, bacterial growth was indicated by adding 50 µL of 0.2 $mgmL^{-1}$ *p*-iodonitro tetrazolium chloride (INT) to all the microtitre plate wells and further incubated at 37 ºC for 1 h. Biologically active organisms were indicated by a pink-redcolour due to the colorless tetrazolium salt reduction, whereas clear wells demonstrated growth inhibition. The experiment was performed in triplicate.

4.2.7. Data analysis

All data were subjected to analysis of variance using SPSS version 24.00 (IBM Corporation Armonk, New York, NY, United States). Data for nematode *in vitro* bioassays were analysed as a two-way ANOVA based on a completely randomized design (to evaluate the interaction between different concentrations and plant extracts). Results for the greenhouse study were analysed as a one-way ANOVA. Where the data were found not to be normal (1-KS), or the variances were not homogenous, discrete nematode data were transformed through $\log_{10}(x + 1)$

(**Gomez and Gomez, 1984**), but untransformed data is reported. Duncan's Multiple Range Test (p<0.05) was performed to separate the means. Graphs were plotted using Graph-Pad Prism 5.

4.3. Results

4.3.1. Egg hatching bioassay

The nematicidal effects of the different concentrations of *C. maxima*, *P. africana*, *P. sidoides*, *C. sylvaticus*, *S. aculeastrum*, *V. colorata*, *S. lancea* and *M. plumbea* extracts in comparison to the untreated control (water) on the egg mass hatching of *M. incognita* was investigated. The results revealed a significant (p<0.05) effect on egg mass hatching after 24, 48 and 72 h exposure of treatments (**Appendix 4.1 and 4.2**). Relatively to the untreated control (0 $mgmL^{-1}$), the inhibitory effect of methanolic extracts on egg hatching was inversely proportional to concentration and exposure time. The number of hatched eggs decreased with increasing concentration (**Table 4.1**). Hence, all treatments at the applied concentrations suppressed egg mass hatching at 24, 48 and 72 h relative to the untreated control (**Table 4.1**). However, at 0.2 $mgmL^{-1}$, crude methanolic extracts of *P. africana* and *V. colorata* showed an exceptional stimulation of egg mass hatching at 24 h by 20% and 27%, respectively. A slightly enhanced inhibition of egg mass hatchability was observed with an increase in crude methanolic extract concentrations. On the other hand, *C. maxima* showed severe suppression of nematode hatchability of egg masses with 44–84%, 21–96%, and 77–96% inhibition after the exposure periods of 24, 48 and 72 h, respectively (**Table 4.1**). Overall, crude methanolic extract of *C. maxima* was the most active at decreasing the emergence of juveniles from egg masses under the *in vitro* bioassay. Water extracts showed opposite results to those obtained with crude methanolic extracts. Egg hatching was very low at 24 h but increased at 48 – 72 h (**Table 4.2**).

Table 4.1. Effect of eight methanolic plant crude extracts on number of hatched juveniles from single egg mass and inhibition impact (%) relative to the untreated control of root-knot nematode, *M. incognita* under *in vitro* conditions.

Plant crude extracts ($mgmL^{-1}$)	24-h		48-h		72-h	
	Mean ±SE	%	Mean ±SE	%	Mean ±SE	%
Untreated control (water)	53.33±16.76^{ab}	0.00	57.72±16.51^{a}	0.00	71.99±16.17^{a}	0.00
P. sidoides						
0.2	34.00±9.94^{c-i}	-36	21.16±3.81^{i-p}	-63	39.83±4.21^{c-f}	-45
0.4	29.50±3.39^{c-k}	-45	19.00±0.27^{j-p}	-67	30.50±1.69^{d-h}	-58
0.6	18.00±1.25^{h-k}	-66	17.83±8.72^{l-p}	-69	28.66±3.31^{f-n}	-60
0.8	8.83±5.86^{jk}	-83	8.00±0.28^{op}	-86	17.16±3.86^{j-p}	-76
P. africana						
0.2	63.83±2.23^{a}	20	42.67±5.91^{b-g}	-26	45.66±4.08^{bc}	-37
0.4	46.33±3.42^{a-e}	-13	30.00±0.72^{d-g}	-48	38.66±4.46^{c-f}	-46
0.6	33.00±3.74^{c-i}	-38	28.50±5.04^{f-n}	-51	18.50±1.52^{e-i}	-74
0.8	27.50±5.56^{c-k}	-49	20.50±4.53^{i-p}	-64	10.17±4.48^{hi}	-86
S. aculeastrum						
0.2	45.00±7.28^{a-f}	-16	40.17±7.48^{b-f}	-30	43.67±5.10^{bc}	-39
0.4	36.50±8.56^{c-i}	-32	35.66±3.86^{c-k}	-38	35.83±4.38^{c-g}	-50
0.6	32.83±4.84^{c-j}	-38	23.00±4.58^{h-p}	-60	23.83±8.53^{e-i}	-67
0.8	22.33±3.67^{f-k}	-58	22.17±1.91^{h-p}	-62	18.33±4.71^{e-i}	-75
V. colorata						
0.2	67.67±9.15^{a}	27	32.33±14.53^{d-k}	-44	45.67±12.68^{bc}	-37
0.4	42.67±2.68^{a-g}	-20	29.50±11.89^{f-n}	-49	32.67±7.62^{d-h}	-55
0.6	29.83±9.63^{c-k}	-44	26.50±14.92^{f-o}	-54	28.83±13.08^{d-h}	-60
0.8	23.00±2.42^{e-k}	-57	28.83±10.65^{c-j}	-67	13.33±8.70^{e-i}	-81
S. lancea						
0.2	47.00±15.02^{a-e}	-12	38.83±8.87^{c-j}	-33	33.50±8.71^{d-h}	-53
0.4	35.67±6.62^{c-i}	-33	32.67±4.49^{e-m}	-43	31.00±6.75^{d-h}	-57
0.6	26.67±8.66^{c-k}	-50	20.00±4.73^{i-p}	-65	21.50±1.87^{e-i}	-70
0.8	21.00±3.32^{g-k}	-61	20.33±2.57^{i-p}	-65	19.83±2.12^{e-i}	-72
M. plumbea						
0.2	34.17±4.06^{c-i}	-36	36.83±6.15^{d-k}	-36	29.50±7.78^{d-h}	-59
0.4	30.00±9.71^{c-k}	-44	34.67±6.84^{d-k}	-40	27.33±7.26^{d-h}	-62
0.6	28.67±10.40^{c-k}	-46	32.33±7.58^{e-m}	-44	22.33±3.07^{e-i}	-69
0.8	19.00±6.71^{h-k}	-64	20.00±9.30^{i-p}	-65	21.33±9.67^{e-i}	-70
C. sylvaticus						
0.2	25.33±1.90^{d-k}	-53	30.33±11.32^{f-n}	-47	26.83±6.43^{e-i}	-63
0.4	22.33±4.78^{f-k}	-58	24.33±6.68^{g-o}	-58	24.83±7.13^{e-i}	-66
0.6	14.83±6.71^{i-k}	-72	34.33±6.64^{d-k}	-41	26.67±1.19^{e-i}	-63
0.8	11.33±2.60^{i-k}	-79	23.00±3.84^{h-p}	-60	16.67±3.34^{e-i}	-77
C. maxima						
0.2	30.16±1.09^{c-k}	-44	45.67±1.44^{a-c}	-21	16.33±4.80^{e-i}	-77
0.4	20.00±0.27^{h-k}	-63	17.83±0.72^{l-p}	-69	11.50±8.91^{g-i}	-84
0.6	13.33±0.72^{i-k}	-75	10.33±00^{n-p}	-82	7.83±0.54^{hi}	-89
0.8	8.83±1.08^{jk}	-84	2.33±1.44^{p}	-96	2.83±0.27^{i}	-96

Column means followed by different letter(s) are significantly different according to Duncan multiple-range test at $p< 0.05$ level; The results are expressed as mean ± standard error; inhibition impact (%) = $\left(\frac{\text{treatment}}{\text{control}} - 1\right)100$.

Table 4.2. Effect of eight water plant crude extracts on number of hatched juveniles from a single egg mass and inhibition impact (%) relative to the untreated control of root-knot nematode, *M. incognita* under *in vitro* conditions.

Plant crude extracts ($mgmL^{-1}$)	24-h		48-h		72-h	
	Mean ±SE	%	Mean ±SE	%	Mean ±SE	%
Untreated control (water)	53.33±16.76^{a}	0.00	57.72±16.51^{a}	0.00	71.99±16.17^{c}	0.00
C. sylvaticus						
0.2	9±2.91^{gh}	-83	25±5.15^{e-k}	-57	33±4.51^{g}	-54
0.4	5±0.88g^{h}	-91	34±2.91^{c-i}	-41	51±1.00^{e}	-29
0.6	2±2.96^{h}	-96	40±4.60^{b-g}	-31	60±8.66^{d}	-17
0.8	3±1.86^{h}	-94	52±4.62^{bc}	-10	61±0.66^{d}	-17
P. africana						
0.2	43±4.98^{bc}	-19	51±9.68^{bc}	-12	31±0.72^{g}	-57
0.4	29±3.56^{b-e}	-46	39±0.13^{c-i}	-32	41±4.91^{f}	-43
0.6	22±2.34^{c-h}	-59	25±6.03^{e-j}	-57	45±2.34^{f}	-37
0.8	23±4.87^{c-h}	-57	26±4.79^{e-j}	-55	76±0.33^{c}	6
S. aculeastrum						
0.2	42±8.89^{b}	-21	25±1.20^{e-j}	-57	24±5.02^{h}	-67
0.4	26±3.57^{b-e}	-51	21±4.96^{f-j}	-64	72±0.33^{c}	0
0.6	14±6.51^{d-h}	-74	17±1.62^{j-l}	-71	92±9.56^{b}	28
0.8	13±3.76^{d-h}	-76	18±4.18^{j-l}	-69	100±6.17^{ab}	39
V. colorata						
0.2	31±2.35^{b-d}	-42	44±6.17^{b-g}	-24	50±3.06^{e}	-31
0.4	4±1.04^{gh}	-94	39±4.96^{c-i}	-32	63±5.00^{d}	-12
0.6	5±2.82^{gh}	-91	33±1.62^{c-i}	-43	84±2.01^{b}	17
0.8	1±2.08^{h}	-98	15±4.18^{j-l}	-74	94±1.86^{b}	31
S. lancea						
0.2	21±6.91^{c-h}	-61	43±9.65^{b-g}	-24	50±0.33^{e}	-31
0.4	25±5.68^{c-h}	-53	47±5.98^{b-g}	-17	50±7.77^{e}	-31
0.6	21±5.91^{c-h}	-61	48±8.74^{b-g}	-17	89±4.27^{b}	24
0.8	11±4.67^{e-h}	-79	36±6.00^{c-i}	-38	126±8.64^{a}	75
M. plumbea						
0.2	10±3.76^{f-h}	-81	10±4.37^{g-l}	-83	31±5.51^{g}	-57
0.4	10±5.20^{f-h}	-81	16±8.19^{g-l}	-72	41±3.11^{f}	-43
0.6	7±3.76^{gh}	-87	13±2.01j^{-l}	-77	56±6.33^{d}	-22
0.8	2±1.00^{h}	-96	3±2.31^{l}	-95	75±10.77^{b}	4
P. sidoides						
0.2	46±4.98^{bc}	-14	44±5.69^{b-g}	-24	42±5.72^{f}	-42
0.4	32±3.56^{d-h}	-40	30±1.76^{d-i}	-48	51±5.91^{e}	-29
0.6	25±2.34^{f-h}	-53	20±9.87^{j-l}	-65	58±2.34^{e}	-19
0.8	15±4.89^{gh}	-72	11±5.69^{j-l}	-81	66±2.33^{c}	-8
C. maxima						
0.2	22±9.50^{c-h}	-59	49±8.05^{b-g}	-15	25±9.17^{h}	-65
0.4	16±5.11^{e-h}	-70	46±3.21^{b-g}	-20	34±0.18^{g}	-53
0.6	5±4.04^{gh}	-91	30±12.85^{d-i}	-48	61±4.44^{c}	-15
0.8	5±1.33^{gh}	-91	14±4.62^{j-l}	-76	119±14.74^{a}	65

Column means followed by different letter(s) are significantly different according to Duncan multiple-range test at $p<0.05$ level; The results are expressed as mean ± standard error; inhibition impact (%) = $\left(\frac{\text{treatment}}{\text{control}} - 1\right)100$.

4.3.2. The effect of plant extracts on J2s penetration

Assessment of the effects of plant extracts on J2s penetration under greenhouse conditions (**Table 4.3**), treatments had highly significant (p<0.01) effects on root gall index and nematode numbers, contributing 93 and 90% in total treatment variation (TTV) of the two variables, respectively (**Appendix 4.3**). Relative to the untreated control, nematode penetration on tomato was highly reduced by *V. colorata* (97%), *S. aculeastrum* (94%), *C. maximum* (89%), *C. sytivaticus* (83%), and *P. africana* (81%) with root gall indices of 0, 3, 2, 3, and 3, respectively (**Table 4.3**). Treatments significantly reduced the invasion and delayed the development of nematodes within tomato roots. There were significantly fewer root galls on tomato roots exposed to egg masses treated with *V. colorata* and *C. maximum* crude extracts as compared to control.

Table 4.3. Influence of plant extracts on nematode penetration on tomato roots after 30 days of inoculation.

	Root gall index		Number of nematodes	
Treatment	**Mean**	**%**	**Mean**	**%**
P. sidoides	3^{b}	-25	53^{b}	-61
P. africana	3^{b}	-25	26^{d}	-81
S. aculeastrum	3^{b}	-25	8^{f}	-94
V. colorata	0^{d}	-100	4^{f}	-97
S. lancea	4^{a}	0	55^{b}	-59
M. plumbea	3^{b}	-25	44^{c}	-67
C. sytivaticus	3^{b}	-25	23^{d}	-83
C. maximum	2^{c}	-50	15^{ef}	-89
Nematode (untreated control)	4^{a}	-	135^{a}	-
Water (without nematodes)	0^{d}	-100	0^{f}	-100
P value	0.00		0.00	

Different letters after the column means indicate significant differences according to Duncan's Multiple Range Test at p<0.05 level. The results are mean ± standard error; inhibition impact (%) = $\left(\frac{\text{treatment}}{\text{control}} - 1\right) 100$.

4.3.2. Juvenile mobility and mortality

Methanol or water extracts of the selected eight botanicals were screened for nematicidal activity against J2s of *M. incognita in vitro*. The juveniles were exposed to 5.0, 2.5, 1.25, 0.625, 0.313 and 0.156 mgmL^{-1} of each plant extract for 24, 48 and 72 h for mobility activity. The results of the current study showed a significant effect of the botanicals and concentration tested (**Appendix 4.4 and 4.5**). Out of the eight plant extracts, two methanolic plant extracts exhibited highly promising immobility activity after 24, 48 and 72 h of exposure (**Table 4.4**). These extracts were from *C. sylvaticus* and *C. maxima* extracts. At the same time, water extracts showed higher mobility inhibition at concentrations within the range of 1.25 to 5 mgmL^{-1}. Seven extracts derived from *P. sidoides*, *P. africana*, *S. aculeastrum, C. sylvaticus, M. plumbea, S. lancea* and *C. maxima* caused relatively high J2s immobility rates ranging from 24% to 100% immobilized J2s after the 72 h exposure period. All plant extracts caused no mortality inhibition on juveniles. When treated J2s were placed in the formulated extracts, immobility was observed, although the juveniles regained mobility when returned to distilled water. This, therefore, showed that the immobilization was temporary.

Table 4.4. Effect of methanol and water plant crude extracts on juvenile immobility rate (%) after 24, 48 and 72 h exposure.

Plant species	Con mg/mL	Water extracts			Methanol extracts		
		24-h	48-h	72-h	24-h	48-h	72-h
Water	0	0. l±0.00^{l}	0.01±0.00^{m}	0.33±0^{o}	0±0.00^{e}	0±0.00^{c}	0±0.00^{d}
P. sidoides	5	20±0.23^{h}	48±1.03^{e}	57±3.12^{ef}	0±0.00^{e}	0±0.00^{c}	0±0.00^{d}
	2.5	0±0.00^{l}	37±1.32^{g}	44±0.54^{h}	0±0.00^{e}	0±0.00^{c}	0±0.00^{d}
	1.25	0±0.00^{l}	0±0.00^{m}	0.33±0.00^{o}	0±0.00^{e}	0±0.00^{c}	0±0.00^{d}
	0.625	0±0.00^{l}	0±0.00^{m}	0±0.00^{o}	0±0.00^{e}	0±0.00^{c}	0±0.00^{d}
	0.313	0±0.00^{l}	0±0.00^{m}	0±0.00^{o}	0±0.00^{e}	0±0.00^{c}	0±0.00^{d}
	0.156	0±0.00^{l}	0±0.00^{m}	0±0.00^{o}	0±0.00^{e}	0±0.00^{c}	0±0.00^{d}
P. africana	5	44±1.56^{d}	50±0.34^{de}	58±3.09^{e}	0±0.00^{e}	0±0.00^{c}	0±0.00^{d}
	2.5	43±2.45^{d}	48±4.21^{e}	50±5.66^{g}	0±0.00^{e}	0±0.00^{c}	0±0.00^{d}
	1.25	16±0.55^{ij}	21±1.87^{ij}	28±1.02^{kl}	0±0.00^{e}	0±0.00^{c}	0±0.00^{d}
	0.625	0±0.00^{l}	0±0.00^{m}	0±0.00^{o}	0±0.00^{e}	0±0.00^{c}	0±0.00^{d}
	0.313	0±0.00^{l}	0±0.00^{m}	0±0.00^{o}	0±0.00^{e}	0±0.00^{c}	0±0.00^{d}
	0.156	0±0.00^{l}	0±0.00^{m}	0±0.00^{o}	0±0.00^{e}	0±0.00^{c}	0±0.00^{d}
S. aculeastrum	5	64±7.09^{b}	85±1.89^{b}	91±5.13^{b}	0±0.00^{e}	0±0.00^{c}	0±0.00^{d}
	2.5	44±0.65^{d}	51±1.07^{de}	55±5.23^{ef}	0±0.00^{e}	0±0.00^{c}	0±0.00^{d}
	1.25	14±1.87^{ijk}	26±2.76^{h}	32±1.56^{jk}	0±0.00^{e}	0±0.00^{c}	0±0.00^{d}
	0.625	0±0.00^{l}	0±0.00^{m}	0±0.00^{o}	0±0.00^{e}	0±0.00^{c}	0±0.00^{d}
	0.313	0±0.00^{l}	0±0.00^{m}	0±0.00^{o}	0±0.00^{e}	0±0.00^{c}	0±0.00^{d}
	0.156	0±0.00^{l}	0±0.00^{m}	0±0.00^{o}	0±0.00^{e}	0±0.00^{c}	0±0.00^{d}
V. colorata	5	0±0.00^{l}	15±1.00^{k}	29±3.98^{kl}	0±0.00^{e}	0±0.00^{c}	0±0.00^{d}

	2.5	0±0.00^{l}	7±3.23^{k}	14±1.05^{m}	0±0.00^{e}	0±0.00^{c}	0±0.00^{d}
	1.25	0±0.00^{l}	0±0.00^{m}	0±0.00^{o}	0±0.00^{e}	0±0.00^{c}	0±0.00^{d}
	0.625	0±0.00^{l}	0±0.00^{m}	0±0.00^{o}	0±0.00^{e}	0±0.00^{c}	0±0.00^{d}
	0.313	0±0.00^{l}	0±0.00^{m}	0±0.00^{o}	0±0.00^{e}	0±0.00^{c}	0±0.00^{d}
	0.156	0±0.00^{l}	0±0.00^{m}	0±0.00^{o}	0±0.00^{e}	0±0.00^{c}	0±0.00^{d}
S. lancea	5	0±0.00^{l}	17±1.56^{jk}	36±2.08^{ij}	0±0.00^{e}	0±0.00^{c}	0±0.00^{d}
	2.5	0±0.00^{l}	0.3l±0.08^{m}	7±3.45^{n}	0±0.00^{e}	0±0.00^{c}	0±0.00^{d}
	1.25	0±0.00^{l}	0.33±0.00^{m}	1±0.00^{o}	0±0.00^{e}	0±0.00^{c}	0±0.00^{d}
	0.625	0±0.00^{l}	0±0.00^{m}	0±0.00^{o}	0±0.00^{e}	0±0.00^{c}	0±0.00^{d}
	0.313	0±0.00^{l}	0±0.00^{m}	0±0.00^{o}	0±0.00^{e}	0±0.00^{c}	0±0.00^{d}
	0.156	0±0.00^{l}	0±0.00^{m}	0±0.00^{o}	0±0.00^{e}	0±0.00^{c}	0±0.00^{d}
M. plumbea	5	33±4.56^{f}	39±4.56^{fg}	56±2.23^{ef}	0±0.00^{e}	0±0.00^{c}	0±0.00^{d}
	2.5	26±2.31^{g}	36±0.55^{g}	45±1.67^{gh}	0±0.00^{e}	0±0.00^{c}	0±0.00^{d}
	1.25	23±2.18^{g}	37±2.45^{h}	42±1.13^{h}	0±0.00^{e}	0±0.00^{c}	0±0.00^{d}
	0.625	12±1.09^{k}	19±2.05^{ij}	26±2.33^{l}	0±0.00^{e}	0±0.00^{c}	0±0.00^{d}
	0.313	0±0.00^{l}	0±0.00^{m}	0±0.00^{o}	0±0.00^{e}	0±0.00^{c}	0±0.00^{d}
	0.156	0±0.00^{l}	0±0.00^{m}	0±0.00^{o}	0±0.00^{e}	0±0.00^{c}	0±0.00^{d}
C. sylvaticus	5	53±1.09^{c}	83±0.88^{b}	95±4.20^{ab}	30±3.56^{c}	97±1.68^{a}	100±0.00^{a}
	2.5	34±1.13^{f}	42±1.56^{f}	66±2.07^{d}	11±1.04^{d}	17±1.03^{b}	38±1.45^{b}
	1.25	12±3.05^{k}	29±1.05^{h}	37±1.07^{i}	0±0.00^{e}	3±0.03c	10±1.03^{c}
	0.625	0±0.00^{l}	0±0.00^{m}	0±0.00^{o}	0±0.00^{e}	0±0.00^{c}	0±0.00^{d}
	0.313	0±0.00^{l}	0±0.00^{m}	0±0.00^{o}	0±0.00^{e}	0±0.00^{c}	0±0.00^{d}
	0.156	0±0.00^{l}	0±0.00^{m}	0±0.00^{o}	0±0.00^{e}	0±0.00^{c}	0±0.00^{d}
C. maxima	5	68±4.54^{a}	96±1.09^{a}	100±0.00^{a}	56±2.03^{a}	100±0.00^{a}	-
	2.5	50±0.52^{c}	63±1.45^{c}	84±0.76^{c}	56±1.00^{a}	100±0.00^{a}	-

1.25	38±0.23^{e}	54±1.06^{d}	63±1.05^{d}	42±0.45^{b}	100±0.00^{a}	-
0.625	0±0.00^{l}	0±0.00^{m}	0±0.00^{o}	0±0.00^{e}	0±0.00^{c}	0±0.00^{d}
0.313	0±0.00^{l}	0±0.00^{m}	0±0.00^{o}	0±0.00^{e}	0±0.00^{c}	0±0.00^{d}
0.156	0±0.00^{l}	0±0.00^{m}	0±0.00^{o}	0±0.00^{e}	0±0.00^{c}	0±0.00^{d}

Column means followed by different letter(s) are significantly different according to Duncan multiple-range test at $p<0.05$ level.

4.3.4. Anti-bacterial activity of the eight medicinal plants on rhizospheric bacteria

The methanolic plant crude extracts of *V. colorata* and *S. lancea* showed the most antibacterial bacterial activity with MIC values ranging from 0.25 to 0.06 µgmL^{-1} and 0.25 to 0.01 µgmL^{-1}, respectively, for eight rhizobacteria (**Table 4.5**). *C. sylvaticus* indicated antibacterial activity at 0.12 µgmL^{-1} on *Pf* and three other isolated rhizobacteria. Furthermore, *P. sidoides*, *S. aculeastrum*, *M. plumbea*, *P. africana* and *C. maxima* crude extracts showed the least bacterial activity with no inhibition effects recorded.

Table 4.5. The antibacterial (Mean Inhibition Concentration values) effects of medicinal plants on rhizospheric bacteria.

	Antibacterial activity MIC (µgmL^{-1})										
Plant extracts	***Pf***	***Bl***	***RT1***	***RT2***	***RT3***	***RT4***	***RT5***	***RT6***	***RT7***	***RT8***	***RT9***
P. sidoides	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
P. africana	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
S. aculeastrum	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
V. colorata	**0.13**	**0.06**	**0.06**	**0.06**	0.25	**0.12**	**0.12**	**0.12**	**0.12**	0.25	**0.06**
S. lancea	**0.13**	**0.01**	**0.01**	**0.03**	0.25	**0.03**	**0.03**	**0.12**	**0.12**	0.25	**0.01**
M. plumbea	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
C. sylvaticus	**0.12**	0.25	0.25	**0.12**	**0.12**	0.25	0.25	0.25	**0.12**	0.25	0.25
C. maxima	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
10% DCMO	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
Neomycin (µgmL^{-1})	**0.008**	**0.0019**	**0.0019**	**0.01**	0.25	**0.0019**	**0.0019**	0.25	**0.0039**	**0.06**	**0.031**

Bold values indicate stimulation activity at those concentrations, thereby suggesting inhibition activity at higher concentrations of those plants, while non-bold values showed no evidence of inhibition on the bacterial strains studied.

MIC = Minimum inhibition concentration; *Pf* = *Pseudomonas fluorescens* ATCC 12525; *Bl* = *Bacillus licheniformis* ATCC 12759; *RT1 – RT9* = nine isolated unidentified rhizobacteria isolated from tomato rhizosphere.

4.4. Discussion

4.4.1. Nematicidal effect of the eight botanical crude extracts on egg hatching and juvenile immobility

Management of plant-parasitic nematodes (PPN) is fast becoming a global phenomenon. Hence, the implementation of successful management strategies is important for suppressing PPN population densities in the soil. The use of extracted plant materials with reduced or no adverse environmental complications, compared to synthetic chemicals, represents a promising alternative for the management of PPN. Egg hatch inhibition by increasing concentrations of *C. maxima*, *P. africana*, *P. sidoides*, *C. sylvaticus*, *S. aculeastrum*, *V. colorata*, *S. lancea* and *M. plumbea* in methanolic and water crude extracts were evaluated. From our results, the egg

hatching inhibition was concentration-dependent with methanolic extracts, whereas, in the water extracts, the highest egg hatch inhibition was observed at low concentrations after 48 – 72 h. These results align with earlier findings by **Akyazi** (**2014**), who reported that *Melia azedarach*'s methanolic extracts at 10% concentration significantly reduced egg hatching by 97%, followed by *Sambucus nigra* (92.9%). Similarly, **Haroon et al.** (**2018**) noted a gradual decrease in egg hatching with an increase in extract concentration and exposure time. **Meyer et al.** (**2012**) showed that egg hatching was inhibited by 85% in the highest concentration tested. **Dube and Mashela** (**2016**) also reported the inhibitory properties of aqueous extracts of wild fruit of *C. africanus* on egg hatching of *M. incognita*. The nematistatic and nematicidal activities of aqueous extracts of *Argemone mexicana* against egg hatching and juvenile mortality of *M. incognita* are due to the presence of different plant constituents, which are detrimental to nematode survivability (**Khan et al., 2017**). In the current study, both crude water and methanolic extracts of *C. maxima* and *C. sylvaticus* inhibited J2s mobility, and this inhibition was temporary. Similar findings were reported by **Javed et al.** (**2008**) with extracts of neem leaves and cakes. Also, **Wuyts et al.** (**2006**) reported that out of 36 pure plant extracts, only 12 had mobility inhibition on *M. incognita* and *R. similis;* hence the inhibition effect was reversible.

Our results demonstrate that some of the crude extracts used are within the stimulation concentration range and showed an increase in egg hatching capacity, while others showed inhibition of egg hatching in *M. incognita*. These findings agree with **Mashela et al.** (**2015**)'s observations, where they noted a significant effect of crude plant extracts on the density-dependent growth (DDG) pattern of nematodes. The DDG patterns involve three phases, i.e., stimulation, neutral, and inhibition phases. **Dube and Mashela** (**2016**) further elaborated that

using a limited concentration range could lead to the observation of only one phase of the DDG pattern. In the present study, *M. plumbea*, *V. colorata* and *C. maxima* showed the highest egg hatching inhibition. Interestingly, the three species exhibited trivial to non-detectable quantities of tannins (**Chapter 3**). This agrees with similar findings by **Ncube et al.** (**2011**), **Muchirah et al.** (**2018**) and **Guenne et al.** (**2011**), who reported insignificant quantities of tannins in *M. plumbea*, *C. maxima* and *V. colorata,* respectively. Additionally, **Sparg et al.** (**2002**) noted the nematicidal efficiency of *Scilla natalensis* (now known as *Merwilla plumbea*) on *Caenorhabditis elegans*. Studies have shown that *Vernonia amygdalina* is highly toxic against *M. incognita* (**Oyedunmade et al., 2009; Abolusoro et al., 2019**). On the other hand, *Cucumis myriocarpus* and *C. africanus* contain antagonistic nematicidal compounds (**Mashela et al., 2015**). The current study showed that *V. colorata* reduced *M. incognita* infection of tomato by 97% and reduced root gall index by 100% at 0.8 mg/mL. Thus, thiarubrine C, or a similar compound, may be present in the *V. colorata* extract, and this may explain, at least in part, the nematicidal properties observed for this plant in our study. Similar results by **Meyer et al.** (**2012**) demonstrated that neem (*Azadirachta indica*) affected the penetration and/or development of nematodes in the roots, thereby reducing root gall formation up to 88% at the highest concentration tested.

4.4.2. Antimicrobial properties of the eight plant extracts

The antimicrobial properties of crude methanolic extracts of all tested plants against *B. licheniformis*, *P. fluorescens* and the nine isolated unidentified rhizobacteria were evaluated in the present study. The results showed that *V. colorata*, *S. lancea* and *C. sylvaticus* inhibited some of the microbial species growth in *in vitro*. These findings are congruent with the previous results

reported by **Golly et al.** (**2012**) on bactericidal activities of leaf extracts of *V. colorata* on resistant Gram-positive and Gram-negative germs such as *Staphylococcus aureus* and *Pseudomonas aeruginosa*. This could be due to the presence of bioactive compounds such as vernolide, 11β,13-dihydrovernolide, and vernodalin earlier reported by **Rabe et al.** (**2002**). Also, **Vambe et al.** (**2019**) demonstrated that leaf extracts of *S. lancea* contain potent antibacterial properties against *Enterococcus faecalis* and *S. aureus,* with tetracosanol and nonadecanol being the main antibacterial compounds. **Selowa et al.** (**2010**) reported antibacterial activities of *C. sylvaticus* against *Escherichia coli*, *E. faecalis*, *S. aureus* and *P. aeruginosa*; however, their findings showed moderate inhibition against the tested bacterial strains. **Ndunda** (**2014**) reported similar inhibition results of *C. sylvaticus* against *Bacillus subtillis* with 2-[N-(2-methylbutanoyl)]-N-phenyl-ethylglutarimide being the active compound. Although the plant extracts were tested on beneficial bacteria in the current study, the results obtained could be comparable to their activities against human pathogenic bacterial strains.

4.5. Conclusions

As far as we know, this is the first study on antibacterial and anti-nematicidal effects of extracts from *P. sidoides*, *P. africana*, *S. aculeastrum, V. colorata, C. sylvaticus, M. plumbea, S. lancea* and *C. maxima* on beneficial microorganisms and root-knot nematodes. Nematicidal investigations of these tested medicinal plants suggested that egg inhibition was one of the mechanisms by which they suppress nematode population densities, with *V. colorata* being the most effective when used for soil amendment. Juvenile immobility was concentration-dependent in the tested extracts with different patterns with respect to those of the same extract when eggs were exposed to them; there was no inhibition of J2s mobility at lower concentrations of the

extracts, while J2s mobility was inhibited at higher concentrations. Out of the eight plants tested, *V. colorata, C. sylvaticus* and *S. lancea* inhibited the growth activity of the beneficial rhizospheric bacteria used. The study further demonstrates the significance of these plant extracts that could be of interest in managing pre- and post-harvest crop losses caused by bacterial infections in agriculture.

CHAPTER 5: Screening of *Bacillus licheniformis*, *Pseudomonas fluorescens,* and medicinal plant powdered material on root-knot nematodes under greenhouse conditions

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5.1. Introduction

Tomato (*Solanum lycopersicum*) is the second most cultivated vegetable crop in South Africa, following potatoes, with about 600 000 tonnes (**Moeng, 2019**). The crop constitutes a wide array of critical beneficial minerals and antioxidants, including alpha-lipoic acid, ascorbic acid, lycopene, folic acid, beta-carotene, lutein, vitamin C, vitamin E, and choline (**Raiola et al., 2014**). During its developmental stages, the tomato plant becomes infected with various pests and diseases, which hinder its production in certain areas (**Lange and Bronson, 1981**). Crop diseases are of great economic value causing a considerable loss to the food supply chain (**Savary et al., 2019**). Globally, losses due to crop diseases amount to 16% of all crops lost each year (**https://bigdata.cgiar.org/big-data-collaboration-for-crop-loss-research/**). Tomato is one of the significant crops that are adversely affected by root-knot nematodes.

Agriculture allocates a substantial share of capital revenue to job creation and food security in both developing and developed countries (**Riesgo et al., 2016**). Thus, a significant amount of chemical fertilizer is used to replenish the soil with nitrogen and phosphorus. In the 1960s, for example, the Green Revolution-I was launched to add greater amounts of synthetic chemicals such as fertilizers, pesticides, and herbicides to meet the food demand by the world's growing population (**Popp et al., 2013**). However, other environmental aspects such as soil fertility, soil microbial diversity, surface, and groundwater are adversely affected by the excessive use of

these synthetic chemicals (**Meena et al., 2020**). Globally, persistent plant diseases continue to seriously affect agriculture, resulting in widespread crop losses and decreased yields (**Savary et al., 2012**). Plant-parasitic nematodes (PPNs) minimize the yield of many economically valuable vegetable crops (**Abd-Elgawad, 2014**), with the southern root-knot nematode being the most prevalent PPN (**Koening et al., 1999**), causing a notable significant degree of damage in sandy soils (**Sikora and Fernández, 2005**).

Plant-parasitic nematodes play a significant role as a contributory agent of crop diseases and significantly impact the economy (**Bernard et al., 2017**). *Meloidogyne incognita* is one of the most important parasitic nematodes infecting tomato plants (**Bernard et al., 2017**). Due to the withdrawal of synthetic nematicides and lack of resistance to tomato plants, management of population densities of root-knot nematode has become a significant barrier (**Coyne et al., 2018**). Eco-friendly management measures, including the use of bio-controls, have gained much interest in controlling insects and diseases (**Barratt et al., 2018**). Plant growth-promoting rhizobacteria (PGPR) is one of the micro-organisms used as a biological control in the management of PPNs. PGPR are known to enhance root growth by colonising the rhizosphere (**Bhattacharyya and Jha, 2012; Gouda et al., 2018**). These are a group of free-living bacteria of diverse genera with *Bacillus* and *Pseudomonas* spp. being the most dominant (**Podile and Kishore, 2007**). PGPR release distinct biochemical compounds that can be employed to suppress nematode population densities (**Akhtar and Siddiqui, 2010; Cetintas et al., 2018; Sidhu, 2018; Khanna et al., 2019a**).

Biological solutions are naturally produced in the form of micro-organisms (bacteria or fungi) capable of suppressing phytopathogens and promoting plant growth without significant environmental damage. Hence, microbial inoculants are used to speed up biological activities, consequently increasing the availability of nutrients to crops by fixing atmospheric nitrogen, making insoluble phosphate soluble, and decomposing plant and animal waste material (**Rashid et al., 2016**), resulting in the release of nutrients and antibiotics that are attributed to antagonizing of various soil pathogenic micro-organisms through the production of siderophores, cyanide hydrogen (HCN), chitinase, and β-1,3-glucanase (**Saraf et al., 2014**).

The use of organic soil amendments has been shown to control soil-borne pathogens, including root-knot nematodes (**Mashela, 2002**). Studies have shown that soil amended with organic matter resulted in the stimulation of antagonistic microorganisms, and the liberation of secondary volatile or non-volatile phytochemicals with nematicidal properties (**Ntalli et al., 2020a**). Furthermore, soil amendments have exhibited significant improvement of plant growth resulting in induced plant tolerance and resistance to PPNs (**Chavarria-Carvajal and Rodriguez-Kabana, 1998**). Therefore, in the current study, a series of experiments were conducted to establish the *in vitro* and *in vivo* plant growth-promoting attributes of *Bacillus licheniformis* ATCC 12759 and *Pseudomonas fluorescens* ATCC 13525. It also evaluated the impact of eight powdered plant biomass at four different levels on nematode (*Meloidogyne incognita)* infected tomato plants and integrating *B. licheniformis* or *P. fluorescens* with the eight powdered plant biomass as a management strategy of the root-knot nematode.

5.2. Material and methods

5.2.1. Experiment 1: Plant growth promoting properties of *B. licheniformis* and *P. fluorescens* under *in vitro* and greenhouse conditions

5.2.1.1. Phosphate solubilisation

Bacillus licheniformis (ATCC 12759) and *Pseudomonas fluorescens* (ATCC 13525) were purchased from American Type Culture Collection (ATCC). Strains were revived from storage at −70 ºC by streaking a single Micro bank bead onto the fresh nutrient agar (NA) and incubating the plates at 25±2 ºC in an incubator. Before use, the bacteria isolates were maintained on NA slants and stored at 4 ºC.

The phosphate solubilisation capability of the two bacteria was examined according to **Yadav et al**. (**2016**). Briefly, 10 µL of each freshly prepared bacteria strain were spot inoculated on Pikovskaya's (PVK) agar plates amended with 2% tri-calcium phosphate. The solubilising capacity of each bacterium was determined as phosphate solubilising index (PSI). Concurrently, agar plates were incubated at 27± 2 ºC for 7 days, and the inoculated plates were closely observed for the visibility of clear zones around the bacterial colonies. The experiment was done in triplicate, and PSI was calculated on PVK plates as per the formula of **Premono et al.** (**1996**). PSI. = Colony diameter + Clear zone diameter/Colony diameter.

5.2.1.2. Production of indole-3-acetic acid

The modified methods of **Xinxian et al**. (**2011**) and **Toscano-Verduzco et al.** (**2020**) were employed to determine the IAA production by the two bacterial strains. In brief, 40 µL of each

freshly prepared selected bacterial culture were inoculated in Schott bottles containing 40 mL of LB liquid medium fortified with 5 mgL^{-1} L-tryptophan and incubated at 25±2 ºC on an orbital shaker (180 rpm) for 7 days. After the incubation period, 5 mL of each of the bacterial cultures were transferred into sterile centrifuge bottles and centrifuged at 10,000 rpm for 10 min, and the supernatants were collected. Two milliliters of each isolate supernatant collected was mixed with 4 mL of Salkowsky reagent, and the reaction mixture was incubated in the dark for 30 min. The assay was conducted in triplicate. The production of IAA by the microbial isolates was confirmed by measuring the absorbance of the reaction mixture at 530 nm using a UV spectrophotometer. The concentration of IAA in µg/mL was estimated by using a standard curve: $y = 0.1167x + 0.1686$, $R^2 = 0.9132$ derived from 0,1,5,7, and 10 µg/mL of standard IAA (Sigma) diluted in sterile distilled water.

5.2.1.3. Hydrogen cyanide production

The ability of the selected endophytic bacteria and fungi to synthesise hydrogen cyanide was evaluated based on the method of **Bakker and Schippers** (**1987**). Each bacterial culture was streaked on Petri dishes containing Luria Bertani (LB) agar supplemented with 4.4 gL^{-1} of glycine. In contrast, sterilised Whatman filter paper (No. 1) pre-soaked in 0.5% picric acid and 2% sodium carbonate for 1 min was gently stuck on the lids of each of the Petri dishes. The plates were then sealed with parafilm and incubated for 7 days at 28 ± 2 °C. A change in colour of the filter papers from yellow to deep orange or reddish-brown indicated a positive result. Three replicates were made for each bacterium.

5.2.1.4. Aminocyclopropane-1-carboxylate (ACC) deaminase activity

The bacterial strains were evaluated for their ability to use ACC as their sole source of nitrogen using a protocol of **Jasim et al.** (**2013**) with minor modification. Briefly, the bacterial strains were inoculated on plates containing Dworkin and Foster (DF) minimal salts agar augmented with 3 mM ACC as the only source of nitrogen. In triplicates, the plates were incubated at 28 ± 2 ºC for 5 days, and the manifestation of bacterial growth afterwards was considered a positive result.

5.2.1.5. Siderophore production

Siderophore production was qualitatively investigated by chrome azurol S (CAS) agar for all the selected bacterial isolates using a modified method of **Milagres et al.** (**1999**). CAS-blue agar was prepared by dissolving 60.5 mg of CAS into 50 mL of distilled water mixed with 10 mL of iron (III) solution (1 mM $FeCl_3$. $6H_2O$ in 10 Mm HCl). The mixture was gently mixed under a magnetic stirrer and 72.9 mg of hexadecyltrimethylammonium (HDTMA) bromide dissolved in 40 mL distilled water was added, and the resulting dark-blue mixture was then autoclaved at 121°C for 15 min. Simultaneously, a mixture of 30.24 g PIPES, 15 g of agar, 900 mL of distilled water, and 50% (w/w) of NaOH to adjust the pKa of PIPES to 6.8 was also autoclaved. Finally, the two mixtures were mixed gently and agitated under the laminar flow bench to avoid foaming and, after that, poured aseptically into plates. Upon solidification, freshly prepared selected bacterial cultures were spot inoculated on their respective plates and incubated for 7 days at 25 ± 2 ºC. The appearance of a yellow/orange or purple halo around the microbial colonies was taken as a positive result for siderophore production. The experiment was conducted in triplicate.

5.2.1.6. Production of ammonia

The production of ammonia was investigated using a modified method of **Hassan** (**2017**) and **Passari et al.** (**2016**) using Nessler's reagent in peptone broth. Briefly, in triplicates, each of the freshly prepared bacterial cultures were aseptically inoculated into their respective labeled test tubes containing 10 mL of peptone liquid media, and incubated for 7 days at 28 ± 2 ºC on an orbital shaker at 150 rpm. Thereafter, 1 mL of Nessler's reagent was added to each test tube, and the development of brown to yellow colour indicated a positive test for ammonia production, and absorbance was measured at 530 nm using a spectrophotometer. The concentration of ammonia was evaluated using the standard curve ($y = 1.488x + 0.022$) generated from the standard (ammonium sulfate), and the amount of $(NH_4)_2SO_4$ was expressed in mM.

5.2.1.7. Production of protease enzyme

Protease enzyme production was quantified using Skim Milk Agar (SMA) medium (**Uyar et al., 2011**). The medium was prepared and poured into a Petri dish until solidified under a vacuum hood. Bacteria were grown by streaking on the medium and incubated for 24 h at 25 ± 2 ºC. Positive results were indicated by the presence of clear zones on the surface of the SMA medium.

5.2.1.8. Extraction for phytochemical content and antioxidant activity

The bacteria were inoculated into nutrient broth (NB) and incubated in a rotary shaker for 24 h at 25 ± 2 ºC. After 24 h, the bacteria solution was placed in a separating volumetric flask, and 100 mL of ethyl acetate was added. The flasks were gently shaken and left for a few minutes to allow the bacterial cells to separate from the broth. The broth was drained into a glass beaker; thereafter, the bacterial cells with ethyl acetate were placed in a different glass beaker and

allowed to dry under vacuum and stored in a -4 ºC refrigerator. When needed, 1 mg of the bacterial extract was re-dissolved into a 1 mL 50% aqueous methanol.

5.2.1.9. Determination of total flavonoids content

Flavonoids were determined according to **Makkar** (**1999**) with modifications. Fifty microlitres of *B. licheniformis* and *P. fluorescens* extracts were separately diluted to a volume of 1.0 mL with distilled water. Methanol–HCl (2.5 mL, 95:5 v/v) and catechin reagent (2.5 mL, 1.0 g/100 mL) were added to each reaction mixture and incubated for 20 min at room temperature. A Cary 50 UV–visible spectrophotometer was then used to measure absorbance at 500 nm. As a blank, a reaction mixture containing 50% aqueous methanol was used instead of sample extracts. A pink colouration indicated the presence of flavonoids. The analysis was performed in triplicate, and the concentration of flavonoids in the test samples was estimated using a catechin equivalent (CE) standard curve to calculate mg/g of extract.

5.2.1.10. Determination of total phenolic content

The total phenolic content of the two bacterial species was determined by the Folin-Ciocalteu assay described by **Makkar** (**1999**) using gallic acid as a standard, as described previously (**Chapter 3; Section 3.2.3**).

5.2.1.11. Antioxidant activity

The antioxidant activity of *B. licheniformis* and *P. fluorescens* was evaluated using 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging ability using the modified method of **Liyana-Pathirana and Shahidi** (**2005**). Butylated hydroxytoluene (BHT) was used as a standard in

identifying the antioxidant achievable in terms of the DPPH radical scavenging capability of the bacterial extract. Concentrations ranging from 10-100 µL of each bacterial extract were diluted with 50% aqueous methanol to make up a volume of 1 000 µL and then added to a methanolic $DPPH^{\bullet}$ solution (1 000 µL, 0.1 mM). The $DPPH^{\bullet}$ solution was prepared freshly before the assay. The reaction mixtures were prepared under dim light in test tubes, shaken well, and incubated in the dark for 30 min at room temperature. After incubation, the absorbance was recorded at 517 nm against a blank. The assay was performed in triplicate, and the percentage (%) inhibition of free radical species of the bacterial extracts and BHT was calculated as:

$$\textbf{\% DPPH radical scavenging activity} = \left(\frac{\textbf{absorbance of control} - \textbf{absorbance of test sample}}{\textbf{absorbance of control}}\right) \times \mathbf{100}$$

The 50% inhibition concentration (IC_{50}) for plant extracts was calculated graphically from a linear regression curve by plotting antioxidant percent (%) inhibition versus the corresponding sample concentration.

5.2.1.12. Effect of *B. licheniformis* and *P. fluorescens* on tomato growth within the greenhouse

The study was conducted under greenhouse conditions at the University of KwaZulu-Natal Botanical Garden (290 37.55′ S; 300 24.13′ E), Pietermaritzburg Campus. The day/night temperatures averaged 28/21 ºC, and maximum temperatures were controlled using thermostatically-activated fans on the southern-facing side of the greenhouse and a wet wall on the opposite side to maintain relative humidity at 60–80%. At 0.3 m inter-row and 0.25 m intra-row spacing, eighteen-cm-diameter plastic pots were set on the greenhouse bench. Each pot was filled with 2.5 L steam-pasteurized river sand and vermiculite at a ratio of 2:1 (v/v), and uniform tomato seedlings were transplanted.

Individual colonies of *B. licheniformis* and *P. fluorescens* were picked with a sterile inoculation loop and transferred into 100 mL sterile nutrient broth in 250 mL Erlenmeyer flasks. After 48 h of growing the cultures at 25 °C in an incubator shaker at 180 rpm (revolutions per minute), the solution was transferred into 250 mL centrifuge bottles and centrifuged for 10 min at 1800 rpm. The pellet was collected and was diluted with 100 mL of sterile distilled water. The absorbance of the solution was read at 600 nm using a UV-visible spectrophotometer (Varian Cary 50, Australia) and was applied immediately to the plants. The diluted bacteria (OD 1 at 600 nm) were applied to the soil around the plant stem by a drenching treatment. The application occurred once per week for the first three weeks of the trials. Each plant was watered with 150 mL chlorine-free tap water every other day, and once per week, plants were supplemented with Hoagland's nutrient solution.

5.2.1.13. Determination of photosynthetic pigment

The photosynthetic pigments [total chlorophyll (a+b) and carotenoids] were calculated using **Lichtenthaler's** (**1987**) methodology, as described in **Amoo et al.** (**2014**). Two hundred milligrams of fresh leaf material were ground with 5 mL of ice-cold 80% acetone with a pinch of acid-washed sand (BDH Chemicals Ltd, England). The resulting solution was filtered using a Whatman No. 1 filter paper and centrifuged (Hettich Universal, Tuttlingen, Germany) at 3000 rpm for 10 minutes at room temperature. A UV-visible spectrophotometer (Varian Cary 50, Australia) was used to measure the absorbance of the solution at 470, 645, and 662 nm. The amounts of chlorophyll and carotenoids were calculated as follows:

Chlorophyll a = 11.23A662 – 2.04A645

Chlorophyll b = 20.13A645 – 4.19A662

Chlorophyll a+b = 7.05A662 + 18.09A645

Total carotenoids = (1000A470 –1.90Chla–63.14Chlb)/214

5.2.1.14. Protein estimation

Bovine serum albumin (BSA) was used as a standard to calculate total protein (**Bradford, 1976**). A 200 mg sample was homogenized in 6 mL ice-cold phosphate-buffer saline (PBS) [8 g NaCl (137 mM), 0.2 g KCL (2.7 mM), 1.44 g NA2HPO4 (10 mM), 0.24 g KH2O4 (1.8 mM) in 1 L distilled water (pH 7.2)] using an ice-cold mortar and pestle. The homogenate was centrifuged at 10,000 rpm for 15 min at 4 ºC. A volume of 100 µL of the sample was aspirated and dispersed into a clean test tube, followed by the addition of PBS (Phosphate-buffered saline) (pH 7.4) to make up the volume of 2 mL. All of the test tubes received two milliliters of Bradford dye. The contents of the test tubes were carefully mixed by vortexing and set aside for 5 minutes. The red color changed to blue as Bradford dye bonded to the protein, and the absorbance at 595 nm was measured against a blank using a spectrophotometer.

5.2.1.15. Carbohydrate estimation

With slight adjustments, the total carbohydrate content was calculated according to **Sadasivam and Manickam** (**2008**). Two hundred milligrams of plant material (roots/leaves) were weighed and hydrolyzed in a boiling water bath with 3 mL 2.5 N HCl (hydrochloric acid) for 3 h before being cooled to room temperature. The effervescence was suppressed by adding Na_2CO_3 (sodium carbonate) to the hydrolyzed plant material. Distilled water was added to increase the volume to 5 mL, and the sample was centrifuged at 10,000 rpm for 15 minutes at 4 ºC. One hundred microlitres of the supernatant were taken, and 4 mL of anthrone reagent was added,

followed by the addition of 0.9 mL of distilled water. The test tubes were heated for 8 minutes in a boiling water bath before being promptly cooled under running tap water. As the color developed from green to dark green, the absorbance was measured at 630 nm. Glucose ranging between 0-100 g was used to create the standard curve.

5.2.1.15. Data analysis

Data were subjected to analysis of variance (one-way ANOVA) using SAS (SAS Institute Inc., Cary, NC, USA). Duncan's multiple-range test was used to achieve mean separation. Unless otherwise noted, all treatments discussed were different at a 5% level of probability.

5.2.2. Experiment 2: Impact of powdered plant material on *Meloidogyne incognita* and tomato plant growth

5.2.2.1. Study location/area

The study was conducted under greenhouse conditions as described above (**Section 2.2.1.12**).

5.2.2.2. Experimental design

Four treatments comprised of 0, 2, 6, and 10 g ground of *Cucurbita maxima* (seeds), *Prunus africana* (leaves), *Pelargonium sidoides* (tuberous roots), *Croton sylvaticus* (leaves), *Solanum aculeastrum* (leaves), *Vernonia colorata* (leaves), *Searsia lancea* (leaves) and *Merwilla plumbea* (bulbs) were arranged in a randomised complete block design (RCBD) with six replicates per treatment. Ground plant material were collected and prepared as described in **Chapter 3; Section 3.2.1**. The ground material was stored in sealed plastic bags at room temperature prior to use. On the greenhouse benches, eighteen-cm-diameter plastic pots filled with 2.5 L steam-

pasteurised sand mixed with Hygromix at a ratio of 2:1 (v/v) were placed at 0.3 m inter-row and 0.25 m intra-row spacing. When required, *M. incognita* inoculum was prepared by extracting eggs and second-stage juveniles from the roots of greenhouse-grown nematode-susceptible tomato cv. Roma V.F. in 1% NaOCl (**Hussey and Barker, 1973**).

Four-week-old tomato seedlings (cv. Roma V.F.) were transplanted into pots. A day after transplanting, they were fertilised with 5 g of 2:3:2 (22%) per plant, which provided a total of 310 mg N, 210 mg P, and 260 mg K per mL of water, and 2 g 2:1:2 (43%) of multifeed, which provided a total of 0.35 mg N, 0.32 mg K and 0.32 mg P, 0.9 mg Mg, 0.75 mg Fe, 0.075 mg Cu, 0.35 mg Zn, 1.0 mg B, 3.0 mg Mn and 0.07 mg Mo/mL water (**Mashela, 2002**). A week after transplanting, all plants were inoculated with 2000 *M. incognita* J2s and eggs using a 50-mL plastic syringe, by placing them at 10-cm-radius holes on the cardinal points of the stem of the plants. Plants were treated by dispensing about 0, 2, 6, and 10 g powder of each of the eight plant materials, separately, at 5-cm-radius next to the stem of the plants and mixed well with soil. Every other day, plants were irrigated using a 250 mL beaker of chlorine-free tap water. Plants were monitored for aphids, whiteflies and mealybugs, and sprayed accordingly whenever at least ten aphids per plant were observed.

5.2.2.3 Data collection

Fifty-six days after inoculation, shoot height was measured from the crown to the flag leaf's end and stem diameter using vernier caliper. The shoots were detached from the roots and oven-dried at 52 °C for 72 h and weighed. Roots were removed from pots, rinsed in water to remove soil, blotted dry using filter paper, and weighed. Photosynthetic pigmentation and biochemical

contents were measured as done in **Experiment 1; Section 5.2.1.13 to 5.2.1.15**. Nematodes were extracted from 10 g roots per plant by maceration and blending for 30 sec in 1% NaOCl (**Hussey and Barker, 1973**). The material was passed through nested 150-µm, 75-µm, and 25-µm mesh sieves, with nematodes being collected from the 75-µm and 25-µm mesh sieve. Soil per pot was thoroughly mixed, and a 250 cm^3 soil sample was collected, with nematodes being extracted from soil samples using the sugar-floatation and centrifugation method (**Jenkins, 1964**). Eggs and juveniles were counted from a 10 mL aliquot with the use of a stereomicroscope. Nematode numbers from roots were converted to nematodes per total root system per plant, whereas, nematode numbers (J2s) in soil were converted to 2500 mL soil per pot and were used to determine the final nematode population densities (Pf). The latter was used to compute the reproductive factor (RF = Pf/Pi), a proportion of Pf, and the initial nematode population densities (Pi).

5.2.2.4. Data analysis

Data were subjected to analysis of variance (two-way ANOVA) using SAS (SAS Institute Inc., Cary, NC, USA). Mean separation was achieved through the Duncan's multiple-range test. Unless stated otherwise, treatments discussed were different at a 5% level of probability.

5.2.3. Experiment 3: Effect of *B. licheniformis* and *P. fluorescens* with powdered plant material on nematode infested tomato

5.2.3.1. Location, plant growth conditions, and preparation of materials

The study was conducted under greenhouse conditions, and powdered plant material was collected and prepared as explained previously (**Experiment 1; Section 5.2.1.12**).

5.2.3.2. Experimental design, inoculation, and cultural practices

Treatments comprising of *B. licheniformis* or *P. fluorescens*, with and without powdered plant material, were arranged in a randomised complete block design, with five replicates per treatment. Root-knot nematode (*Meloidogyne incognita*) inoculum was prepared as described previously by **Hussey and Barker** (**1973**). Seven days after transplanting, all plants were inoculated with 2000 *M. incognita* J2s and eggs using a 50-mL plastic syringe, by placing them at 10-cm-radius holes on the cardinal points (around/all directions) of the stem of the plants. Five days before nematode inoculation, 10 g of each eight plant's powdered material were applied individually at 5-cm-radius away from the stem of tomato plants and mixed with soil. The diluted bacteria (OD 1 at 600 nm) of *B. licheniformis* or *P. fluorescens* were applied to the soil around the plant stem as a drenching treatment. The application occurred once per week for the first three weeks of the trials. All cultural practices were done as described in **Experiment 2; Section 5.2.2.2**.

5.2.3.3. Data collection

Plant and nematode data were collected as described in **Experiments 1; Section 5.2.1.13 to 5.2.1.15 and Experiments 2; Section 5.2.2.3.**

5.2.3.4. Data analysis

Data for plant and nematode variables were subjected to analysis of variance using SAS (SAS Institute Inc., Cary, NC, USA) SPPS. Discrete nematode data were transformed through $\log_{10}(x + 1)$ to homogenise the variances (**Gomez and Gomez, 1984**), but untransformed means were reported. Mean separation was achieved through the Duncan multiple-range test at a 5% level of probability. Unless otherwise stated, treatments discussed were significant at 5% level of probability.

5.3. Results

5.3.1. Experiment 1

5.3.1.1. Plant Growth Promoting Properties of *B. licheniformis* and *P. fluorescens*

The two rhizobacterial strains, *B. licheniformis* and *P. fluorescens*, both tested positive for hydrogen cyanide, siderophore production and ethylene production and negative for protease production (**Figure 5.1a-e; Table 5.1**). When phosphate solubilisation was considered the two strains differed in response with *P. fluorescens* testing positive, whereas *B. Licheniformis* showed a negative response (**Figure 5.1a; Table 5.1**). There were statistically significant differences between the two rhizobacteria on secondary metabolite production, with *P. fluorescens* producing significantly higher IAA, phosphate and ammonia when compared to *B. licheniformis*, whereas *B. licheniformis* had significantly higher flavonoids (74.83 mg CEg^{-1}) and phenolics (44.96 mg $GAEg^{-1}$) (**Table 5.2**).The two bacteria strains showed a concentration-dependent DPPH radical scavenging activity with poor IC_{50} values of 210.00 and 191.08 μgmL^{-1} respectively, higher than that of BHT (**Table 5.3**).

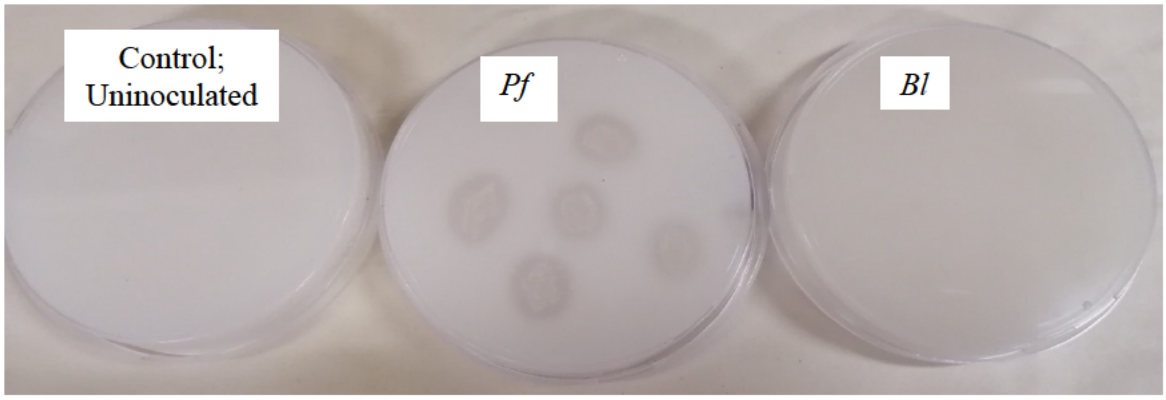

Figure 5.1a. Plates showing *B. licheniformis* (*Bl*) and *P. fluorescens* (*Pf*) phosphorus solubilization.

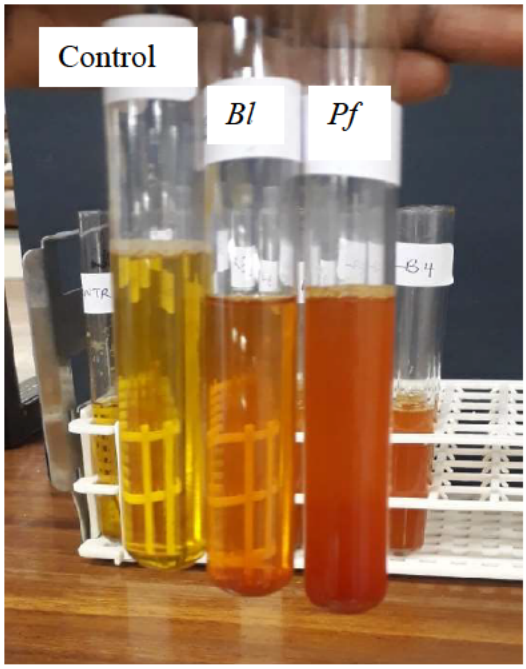

Figure 5.1b. Showing *B. licheniformis* (*Bl*) and *P. fluorescens* (*Pf*) ammonia production.

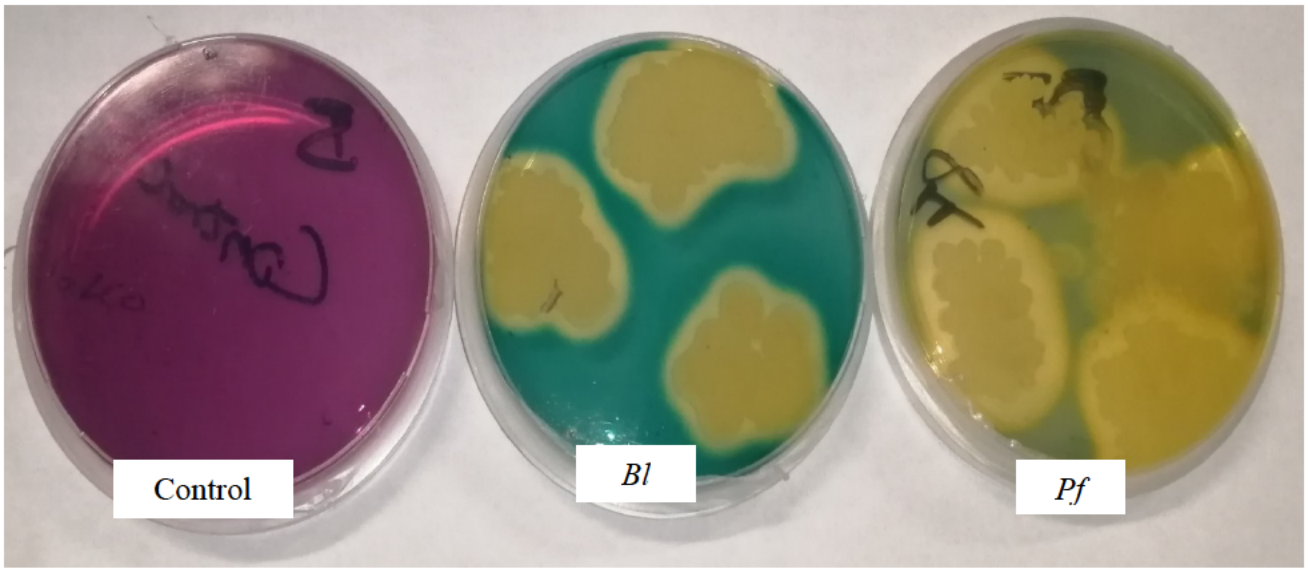

Figure 5.1c. Plates showing *B. licheniformis* (*Bl*) and *P. fluorescens* (*Pf*) siderophore production.

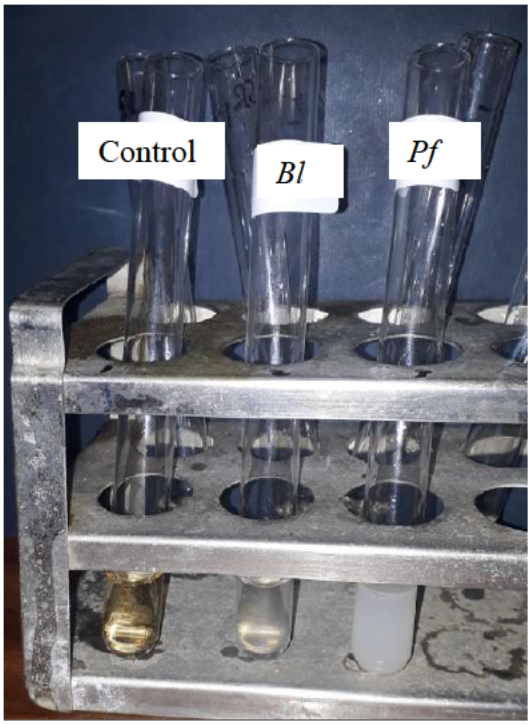

Figure 5.1d. Showing *B. licheniformis* (*Bl*) and *P. fluorescens* (*Pf*) IAA production.

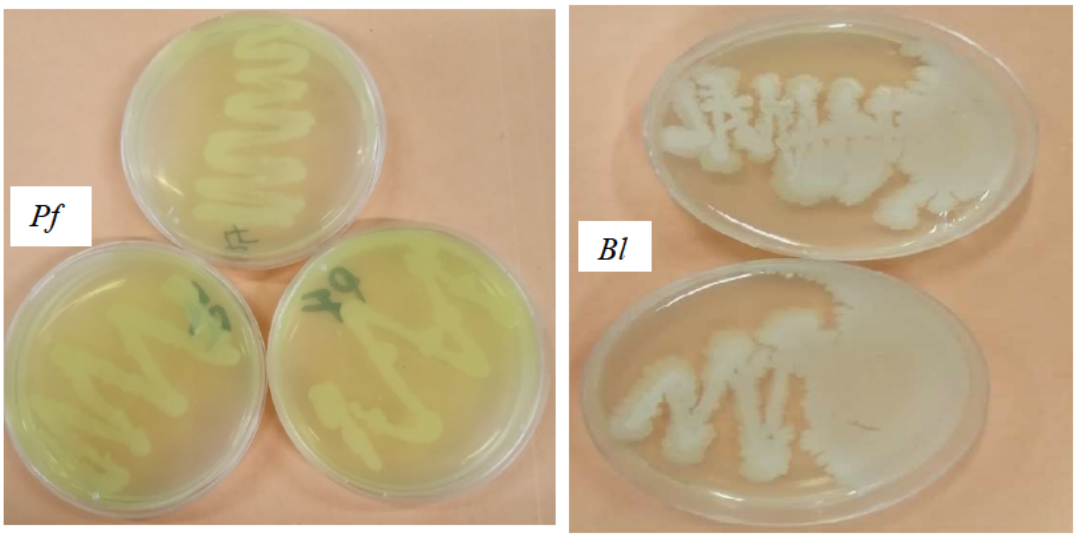

Figure 5.1e. Plates with *B. licheniformis* (*Bl*) and *P. fluorescens* (*Pf*) showing the absence of protease production.

Table 5.1. Plant growth-promoting properties of *B. licheniformis* (*Bl*) and *P. fluorescens* (*Pf*).

Bacteria attributes	***Pf***	***Bl***
Hydrogen cyanide	+	+
Phosphate solubilization	+	-
Siderophore production	+	+
Protease production	-	-
Ethylene production	+	+

WHERE + = Presence and - = Absent

Table 5.2. Plant growth-promoting properties and secondary metabolites of *B. licheniformis* (*Bl*) and *P. fluorescens* (*Pf*).

Bacteria attributes	***Pf***	***Bl***
IAA (μgmL^{-1})	5.15 ± 0.03^{a}	1.49 ± 0.05^{b}
Phosphate (PSI)	2.55 ± 0.07^{a}	2.04 ± 0.08^{b}
Ammonia (NH_3) (mM)	1.87 ± 0.06^{a}	1.21 ± 0.12^{b}
Flavonoids (mg CEg^{-1})	12.97 ± 0.03^{b}	74.83 ± 0.77^{a}
Phenolics (mg $GAEg^{-1}$)	31.92 ± 0.40^{b}	44.96 ± 0.46^{a}

[Y]Means ± SE are averages of three replicates. Means followed by the same letter(s) are not significantly different at $p\leq0.05$ according to LSD test.

Table 5.3. Antioxidant activity of *B. licheniformis* and *P. fluorescens* rhizobacteria compared to a positive control butylated hydroxytaluene.

Concentrations (μgmL^{-1})	***Pf***	***Bl***	**BHT**
10	2.11 ± 0.55^{b}	5.35 ± 0.32^{b}	15.35 ± 0.39^{a}
25	2.11 ± 0.25^{b}	10.35 ± 0.37^{b}	39.83 ± 0.89^{a}
50	9.62 ± 0.42^{b}	17.76 ± 0.35^{b}	68.62 ± 1.02^{a}
100	23.31 ± 0.19^{b}	27.76 ± 0.51^{b}	86.21 ± 0.49^{a}
LC_{50} (μgmL^{-1})	210.00 ± 1.24^{a}	191.08 ± 1.56^{a}	42.98 ± 0.78^{b}

Means followed by same letter(s) are not significantly ($p\leq0.05$) different according to LSD. *Pf* = *Pseudomonas fluorescens*; *Bl* = *Bacillus licheniformis*; BHT = Butylated hydroxytaluene.

5.3.1.2. Effect of *P. fluorescens* and *B. licheniformis* on growth parameters of tomato plants

The effects of *B. licheniformis* and *P. fluorescens* were not significant ($p \leq 0.05$) on stem diameter, root length, fresh shoot weights, but had effects on plant height, dry shoot weight and fresh root weight, with treatments contributed 79, 64 and 63% in total treatment variation (TTV), respectively (**Appendix 5.1**). The growth of tomato was considerably influenced by *B. licheniformis* and *P. fluorescens* compared to the untreated control (**Table 5.4**). A significant increase was noted in plant height after applying *B. licheniformis* and *P. fluorescens* alone and combined. The overall root weight of the tomato was enhanced by *Bl* (43%) and *Pf*+*Bl* (40%) relative to the control. Overall, *B. licheniformis* and *P. fluorescens* combined, enhanced several plant variables. Furthermore, *P. fluorescens* alone significantly induced chlorophyll and carotenoid content production compared when used combined with *B. licheniformis* (**Figure 5.2**). Furthermore, treatments had no effect on leaf carbohydrate content, but B. licheniformis administered alone increased root carbohydrate content and leaf protein levels when compared to the control; however, the roots of control tomato seedlings had higher protein content than the roots of treated tomato seedlings (**Figure 5.2**).

Table 5.4. Effect of *Pseudomonas fluorescens* (*Pf*) and *Bacillus licheniformis* (*Bl*) on growth parameters of tomato seedlings after 30-days of bacterial inoculation under greenhouse conditions.

Treatment	Shoot height (cm)		Root length (cm)		Stem diameter (mm)		Fresh shoot weight (g)		Fresh root weight (g)		Dry shoot weight (g)		Dry root weight (g)	
	[Y]Mean	[Z]%	Mean	%	Mean	%	Mean	%	Mean	%	Mean	%	Mean	%
Pf	59.23±0.36[a]	23	18.59±0.18[a]	-11	7.40±0.15[a]	12	17.46±0.37[a]	-5	9.83±0.37[b]	-2	2.77±0.17[a]	-29	0.83±0.09[b]	-1
Bl	54.87±0.46[ab]	14	19.49±0.13[a]	-6	6.46±0.41[a]	-2	20.72±0.30[a]	13	14.25±0.35[a]	43	2.94±0.17[a]	-15	1.20±0.08[a]	43
Pf+*Bl*	59.60±0.48[a]	24	23.74±0.20[a]	14	7.15±0.41[a]	8	19.58±0.28[a]	7	13.96±0.31[a]	40	3.52±0.14[a]	-10	1.18±0.07[a]	41
Control	48.22±0.45[b]	-	20.79±0.15[a]	-	6.63±0.45[a]	-	18.38±0.39[a]	-	9.99±0.38[b]	-	3.89±0.12[a]	-	0.84±0.09[b]	-

[Y]Means ± SE are averages of six replicates. Column means followed by the same letter(s) are not significantly different at $p \leq 0.05$ according to Waller-Duncan Multiple Range test. Impact (%) = $\left(\frac{\text{treatment}}{\text{control}} - 1\right) 100$. Percentage values with a negative sign show a decrease in plant growth parameters, while those without a negative sign indicate an increase in the measured parameter.

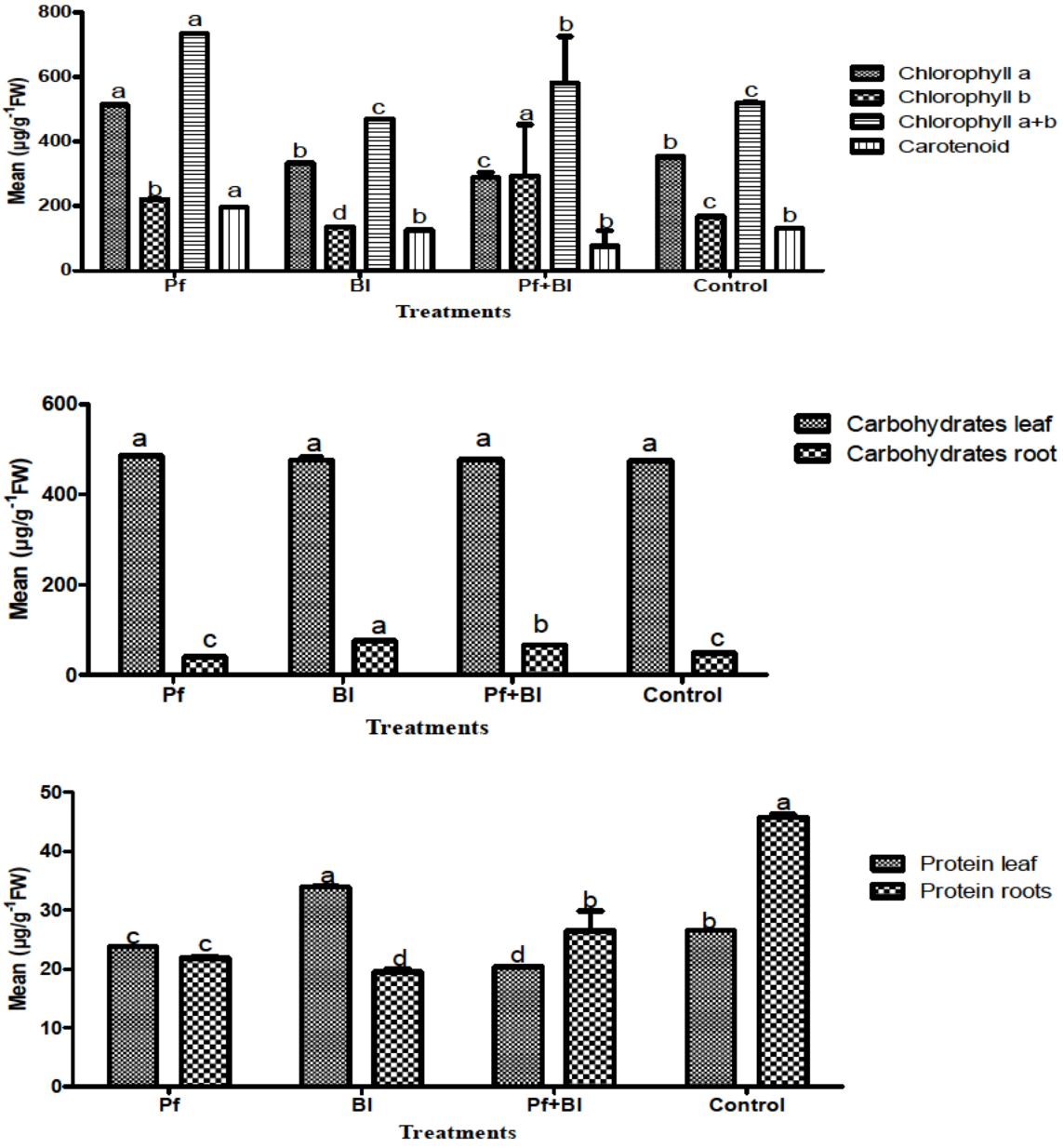

Figure 5.2. Effect of *Pseudomonas fluorescens* and *Bacillus licheniformis* on chlorophyll, carotenoid, carbohydrate, and protein content of tomato plant. Means ± SE are averages of six replicates. According to the Waller-Duncan multiple-range test, bar means followed by the same letter(s) are not significantly different at $p \leq 0.05$.

5.3.2. Experiment 2

5.3.2.1. Effect of botanicals on nematode-infected tomato plant growth parameters

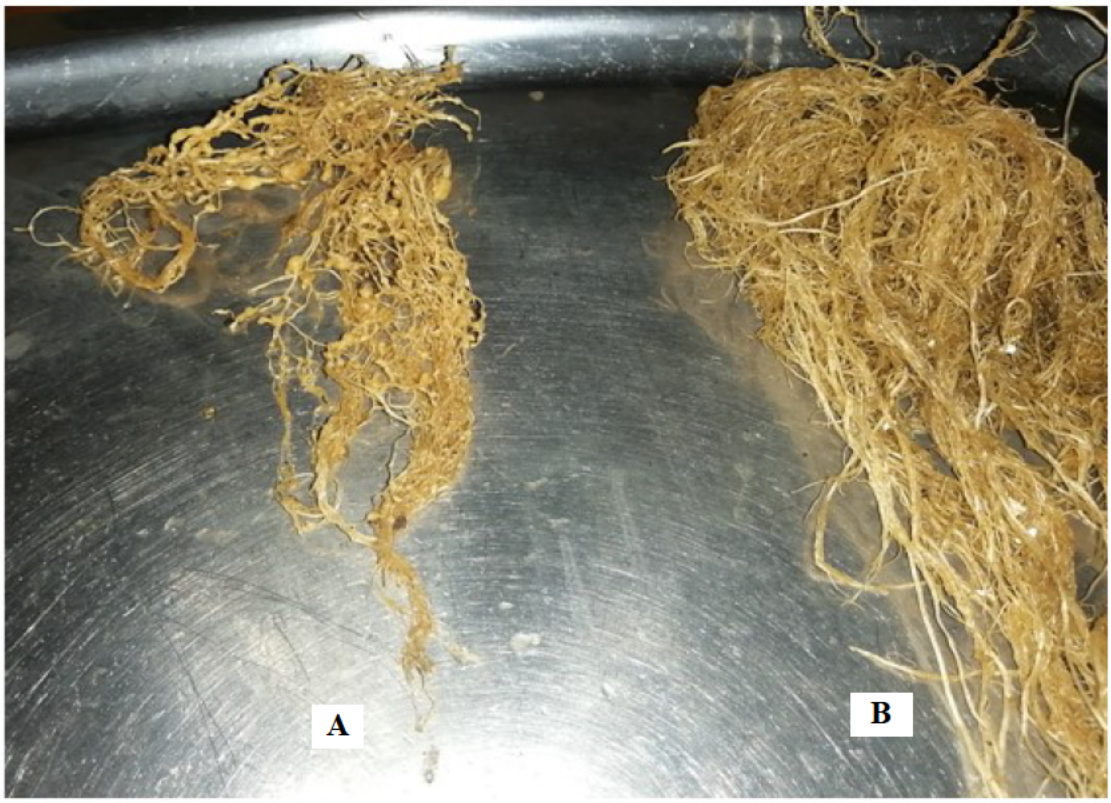

Figure 5.3. A = Stunted tomato root growth due to root-knot nematode infection and B = Healthy roots.

The application of botanicals at different levels had a significant (p<0.05) effect on shoot height, stem diameter, root length, fresh shoot weight, fresh root weight and dry shoot weight (**Appendix 5.2**). *Croton sylvaticus* and *Cucurbita maxima* at 10 g significantly improved plant height comparable to the uninoculated plants (**Table 5.5**). Relative to the untreated control, the eight medicinal powdered plant material at all application rates significantly enhanced the plant height of tomato seedlings; except plants treated with 2 g and 6 g of *S. lancea* and *P. sidoides*. On the other hand, comparable to the uninoculated plants, the root length of treated tomato plants

was significantly reduced (**Figure 5.3 to 5.5**). However, treatments improved the root length of treated plants compared to the untreated-inoculated controls, except for plants treated with *S. lancea* at the lowest rate. It was noted with the stem diameter of treated tomato plants, soil amended with 10 g of *S. aculeastrum*, *V. colorata*, *C. sylvaticus* and *C. maxima* had stem diameter larger or equal to those of the uninoculated plants. The fresh and dry shoot weight per plant varied among soil amendments and plant species (**Table 5.5**). The amended soil treatments had significantly higher fresh shoot weight per plant than the untreated control. However, the findings were substantially lower than the uninoculated plants. Lower, medium and higher levels of *M. plumbea*, *C. maxima* and *C. sylvaticus* amendments gave corresponding higher fresh/dry shoot weight per treatment. The fresh root weight per plant grown on the amended potted soil with *M. plumbea*, *C. maxima* and *C. sylvaticus* was also significantly higher than those grown on the untreated potted soil. Higher levels of amendments resulted in enhanced root weight and longer root length of the tomato plants relative to the untreated potted soil.

5.3.2.2. The effect of botanicals on the total chlorophyll and carotenoid content on nematode-infested tomato plant

The total chlorophyll and carotenoid contents in the tomato leaves increased with the increasing application rate of the eight tested soil amendments (**Figure 5.6**). Relative to the untreated potted soil, 10 g of the amendment significantly improved chlorophyll a, b, and a+b contents with a slight decreased noted on tomato leaves treated with *S. aculeastrum*. Similar values were observed with carotenoid contents. The highest carotenoid contents were produced on leaves of plants grown in soil amended with *C. maxima* and *C. sylvaticus*. At the same time, *S.*

aculeastrum, *V. colorata* and *S. lancea* produced slightly lower or equal to carotenoid contents of those of untreated plants.

5.3.2.3. Effect of botanicals on the estimated total carbohydrates and protein contents of the nematode-infected tomato plant

The total carbohydrate contents in both the tomato leaves and roots increased with the increasing rates of the soil amendments (**Figure 5.8**). The differences in the carbohydrate contents in both the tomato leaves and roots were significant ($p \leq 0.05$) among the treatments. **Figure 5.8** shows that the total carbohydrate contents in the tomato leaves/roots increased relative to the increasing amendment levels in the soil, with the highest carbohydrate contents produced on plants treated with *C. sylvaticus.* The total protein contents in both leaves and roots were significantly increased with the application of 2 g and 6 g of soil amendments (**Figure 5.7**).

5.3.2.4. Effect of botanicals on the estimated flavonoids and total phenolics contents of the nematode-infected tomato plant

The influence of organic soil amandments at different levels (2, 6 and 10 g) and nematode infection on the flavonoids and total phenolic content of tomato plants are shown in **Table 5.6**. Compared to the uninoculated plants, the results of the present study show an increase in flavonoids and total phenolic content in the untreated plants by 7 and 7%, respectively. The levels of total phenolic content showed a significant increase in the plants treated with 2 g of organic amendments compared with the untreated plants except for plants treated with *M. plumbea* and *C. maxima*. Furthermore, nematode-infected plants treated with *P. sidoides*, *P. africana*, *V. colorata* and *C. sylvaticus* at 10 g had a decreased total phenolic content by 1, 5, 30

and 40%, respectively. Relative to the untreated plants, the result further showed that flavonoids content was increased by 105, 23 and 14% with *P. sidoides* at 2, 6 and 10 g, respectively. Total flavonoids content was also increased by 27 and 51% with *S. lancea* and *C. sylvaticus* treatments at 10 g, respectively, in the infected plants.

5.3.2.5. Effect of botanicals on nematode parameters

Figure 5.3 show the prevalence of root-knot nematodes infection on tomato roots. Treatments had a significant effect on the number of eggs and J2s, with botanicals contributing 100 and 89% to the total treatment variation, respectively (**Appendix 5.3**). The nematode numbers of infected tomatoes decreased as the levels of soil amendments increased in the potted soil (**Table 5.7**). However, the numbers of eggs and J2s nematodes in the soil amended with even 2 g plant material were significantly lower than the equivalent numbers in untreated soil, for all treatments. The difference between the 6 g and 10 g treatments, in most cases, was not significant different. The lowest nematode numbers were recorded on roots treated with *S. lancea*, *C. maxima*, *V. colorata*, *C. sylvaticus* and *M. plumbea*. Hence, in almost all nematode variables, the product had lower nematode numbers than the untreated control, while at all levels; the product did not induce different effects.

Figure 5.4. Effect of powdered plant material at different levels on tomato root development and nematode infestation relative to the uninoculated control (water). A = *C. maxima,* B = *C. sylvaticus*, C = *M. plumbea* and D = *S. lancea*.

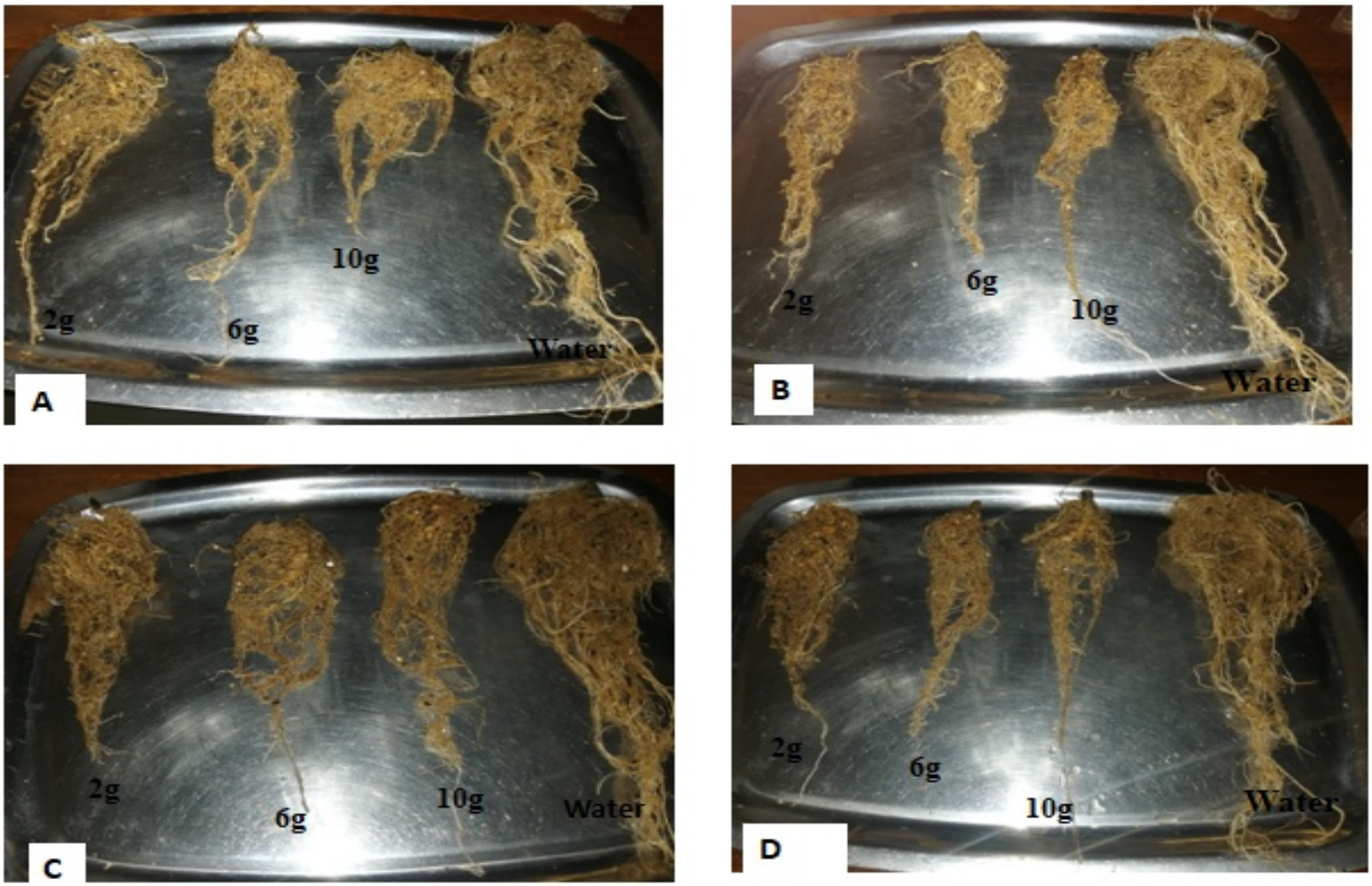

Figure 5.5. Effect of powdered plant material at different levels on tomato root development and nematode infestation relative to the uninoculated control (water). A = *P. sidoides,* B = *P. africana*, C = *S. aculeastrum* and D = *V. colorata*.

Table 5.5. Effect of soil amendments on plant height (PHT), stem diameter (SDM), root length (RTL), fresh shoot weight (FSW), fresh root weight (FRW) and dry shoot weight (DSW) of tomato plant at 56-days after inoculation with 2 000 J2s and eggs.

Plant species	Trt (g)	PHT (cm)		SDM (mm)		RTL (cm)		FSW (g)		FRW (g)		DSW (g)	
		[Y]Means	[Z]%	[Y]Means	[Z]%	[Y]Means	[Z]%	[Y]Means	[Z]%	[Y]Means	[Z]%	[Y]Means	[Z]%
No infection		65.46±0.31^{b-d}	21	8.63±0.15^{a}	35	41.42±0.30^{a}	133	49.41±0.24^{a}	112	30.32±0.28^{a}	221	8.47±0.07^{a}	278
Nematodes only		54.01±0.39^{j-l}	-	6.41±0.09^{gh}	-	17.78±0.24^{gh}	-	23.29±0.32^{jk}	-	9.45±0.27^{mn}	-	2.13±0.12^{mn}	-
P. sidoides	2	52.10±0.31^{kl}	-4	5.99±0.08^{i}	-7	18.60±0.25^{d-f}	5	21.31±0.22^{kl}	-9	14.83±0.20^{g-k}	5	2.22±0.07^{mn}	4
	6	58.94±0.19^{e-h}	9	6.65±0.12^{gh}	4	22.84±0.28^{gh}	29	25.75±0.42^{ij}	11	14.53±0.29^{h-k}	54	3.79±0.18^{h-j}	78
	10	64.56±0.29^{b-d}	20	7.09±0.03^{e-g}	11	27.66±0.40^{b}	56	31.74±0.24^{fg}	36	15.94±0.27^{f-i}	69	2.78±0.17^{j-n}	31
P. africana	2	54.5±0.38^{i-k}	1	6.51±0.18^{f-h}	2	22.50±0.44^{ef}	27	26.34±0.32^{ij}	13	7.40±0.25^{n}	-22	2.22±0.13^{mn}	4
	6	58.06±0.23^{f-j}	8	6.91±0.11^{h}	8	27.26±0.38^{bc}	53	26.84±0.43^{h-j}	15	10.49±0.36^{lm}	11	3.76±0.18^{h-j}	77
	10	62.04±0.43^{d-f}	15	7.78±0.10^{cd}	21	28.34±0.23^{b}	59	24.37±0.22^{jk}	5	14.21±0.22^{h-k}	50	3.16±0.13^{j-n}	48
S. aculeastrum	2	56.30±0.19^{g-j}	4	7.36±0.08^{d-f}	15	21.48±0.21^{fg}	21	28.61±0.20^{g-i}	23	16.13±0.32^{f-i}	71	2.78±0.10^{j-n}	31
	6	58.00±0.11^{f-j}	7	8.62±0.19^{a}	35	25.54±0.51^{b-d}	44	34.81±0.33^{ef}	50	18.90±0.19^{d-f}	100	3.66±0.05^{ij}	72
	10	58.46±0.25^{f-i}	8	8.76±012^{a}	37	26.62±0.31^{b-e}	50	36.31±0.38^{de}	56	16.65±0.38^{f-h}	76	3.17±0.15^{j-n}	49
V. colorata	2	55.12±0.22^{h-k}	2	6.52±0.12^{h}	2	21.60±0.34^{c-f}	22	19.25±0.31^{l}	-17	13.39±0.28^{i-l}	42	2.55±0.17^{j-n}	20
	6	58.20±0.43^{f-i}	8	8.09±0.11^{bc}	26	23.98±0.34^{b-f}	35	26.50±0.17^{h-j}	14	13.95±0.31^{h-k}	48	2.69±0.06^{j-n}	26
	10	66.54±0.27^{bc}	23	8.72±0.09^{a}	36	24.36±0.27^{e-g}	37	30.23±0.22^{gh}	30	14.08±0.24^{h-k}	49	3.68±0.10^{h-j}	73

[Y]Means ± SE are averages of five replicates. Column means followed by the same letter(s) are not significantly different at $p \leq 0.05$ according to Waller-Duncan Multiple Range test. [Z]Impact (%) = $\left(\frac{\text{treatment}}{\text{control}} - 1\right)100$. Percentage values with a negative sign show a decrease in plant growth parameters, while those without a negative sign indicate an increase in the measured parameter.

Continuation of Table 5.5.

Plant species	Trt (g)	PHT (cm)		SDM (mm)		RTL (cm)		FSW (g)		FRW (g)		DSW (g)	
		[Y]Means	[Z]%	[Y]Means	[Z]%	[Y]Means	[Z]%	[Y]Means	[Z]%	[Y]Means	[Z]%	[Y]Means	[Z]%
S. lancea	2	49.96±0.51^{l}	-8	6.67±0.09^{gh}	4	17.18±0.18^{h}	-3	24.32±0.33^{jk}	4	11.99±0.22^{k-m}	27	3.05±0.09^{j-n}	43
	6	51.96±0.46^{kl}	-4	7.33±0.13^{d-f}	14	22.02±0.30^{ef}	24	23.45±0.37^{jk}	1	12.39±0.39^{j-m}	31	2.46±0.17^{mn}	15
	10	59.6±0.42^{e-g}	10	7.63±0.11^{cd}	19	25.54±0.50^{b-e}	44	26.18±0.17^{ij}	12	16.67±0.36^{f-h}	76	2.76±0.09^{j-n}	30
M. plumbea	2	55.96±0.21^{g-k}	4	6.64±0.09^{gh}	4	24.18±0.28^{c-f}	36	31.26±0.35^{fg}	34	15.09±0.33^{g-j}	60	3.64±0.12^{h-j}	71
	6	58.36±0.28^{f-i}	8	7.05±0.04^{e-g}	10	25.36±0.29^{b-f}	43	38.92±0.53^{cd}	67	17.87±0.24^{e-g}	89	4.01±0.19^{gh}	88
	10	64.14±0.24^{c-d}	18	7.80±0.11^{cd}	22	28.26±0.48^{b}	59	41.96±0.32^{bc}	80	20.17±0.42^{e-e}	113	5.59±0.11^{cd}	162
C. sylvaticus	2	58.64±0.36^{f-h}	9	6.52±0.07^{h}	2	21.36±0.15^{fg}	20	37.78±0.39^{de}	62	17.72±0.30^{e-g}	88	4.45±0.10^{fg}	109
	6	59.76±0.25^{e-g}	11	7.73±0.08^{cd}	21	22.46±0.22^{ef}	27	43.28±0.32^{b}	86	21.35±0.19^{b-d}	126	6.21±0.11^{c}	192
	10	68.20±0.20^{ab}	26	8.45±0.10^{ab}	32	23.86±0.28^{c-f}	34	44.04±0.29^{b}	89	22.15±0.18^{bc}	134	6.23±0.20^{c}	192
C. maxima	2	59.00±0.30^{e-h}	9	7.49±0.12^{de}	17	21.90±0.27^{ef}	23	43.87±0.23^{b}	88	24.30±0.34^{b}	157	4.87±0.13^{ef}	129
	6	62.90±0.24^{b-d}	16	7.67±0.10^{cd}	20	22.56±0.32^{ef}	27	44.82±0.17^{b}	92	27.87±0.20^{a}	195	5.77±0.10^{cd}	171
	10	71.32±0.35^{a}	32	8.38±0.09^{ab}	31	24.18±0.30^{c-f}	36	45.30±0.20^{b}	95	28.28±0.25^{a}	199	6.93±0.11^{b}	225

[Y]Means ± SE are averages of five replicates. Column means followed by the same letter(s) are not significantly different at $p \leq 0.05$ according to Waller-Duncan Multiple Range test. [Z]Impact (%) = $\left(\frac{\text{treatment}}{\text{control}} - 1\right) 100$. Percentage values with a negative sign show a decrease in plant growth parameters, while those without a negative sign indicate an increase in the measured parameter.

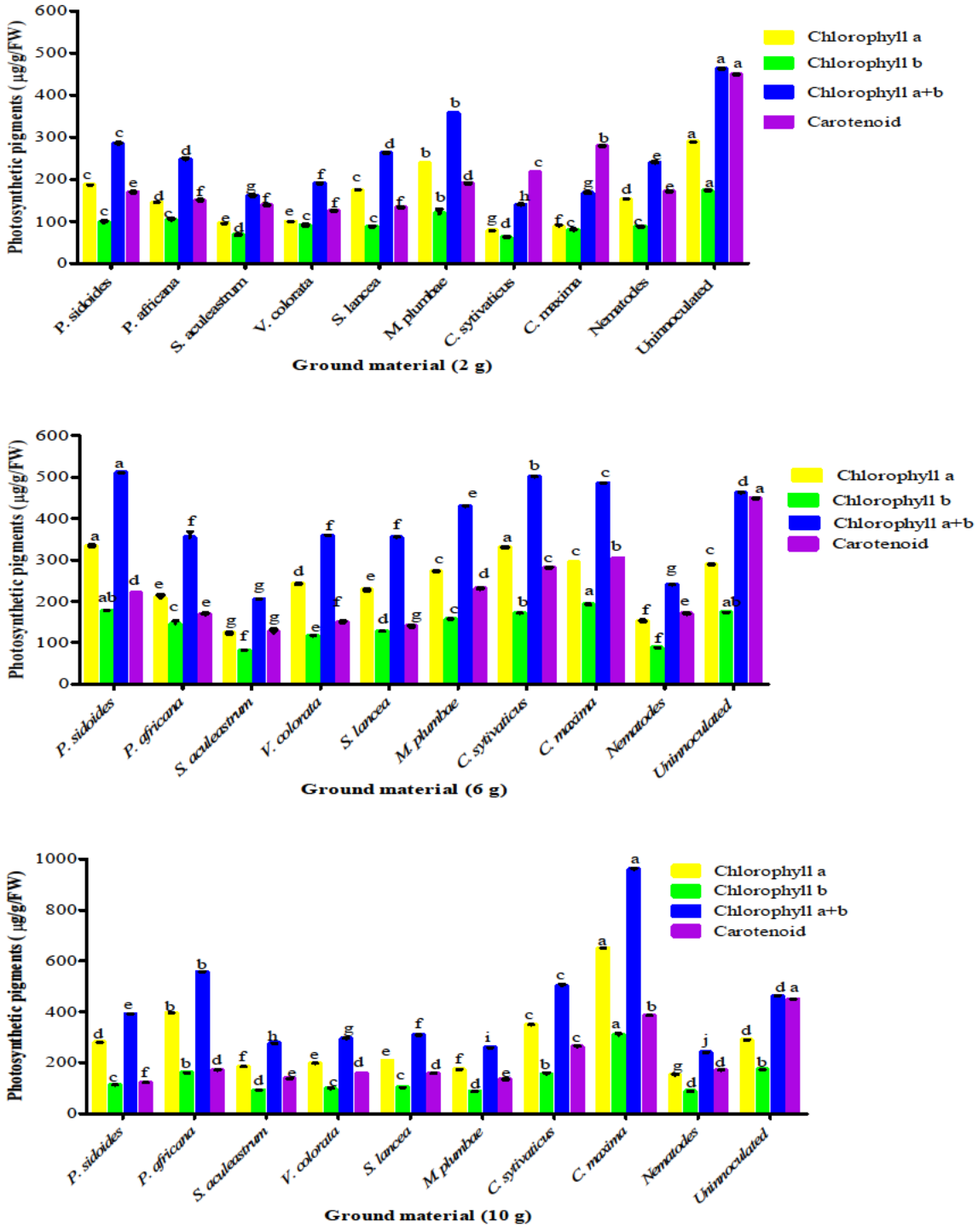

Figure 5.6. Effect of powdered plant material at three application levels on chlorophyll and carotenoid contents of tomato seedlings infected with *M. incognita* relative to the untreated and uninoculated control.

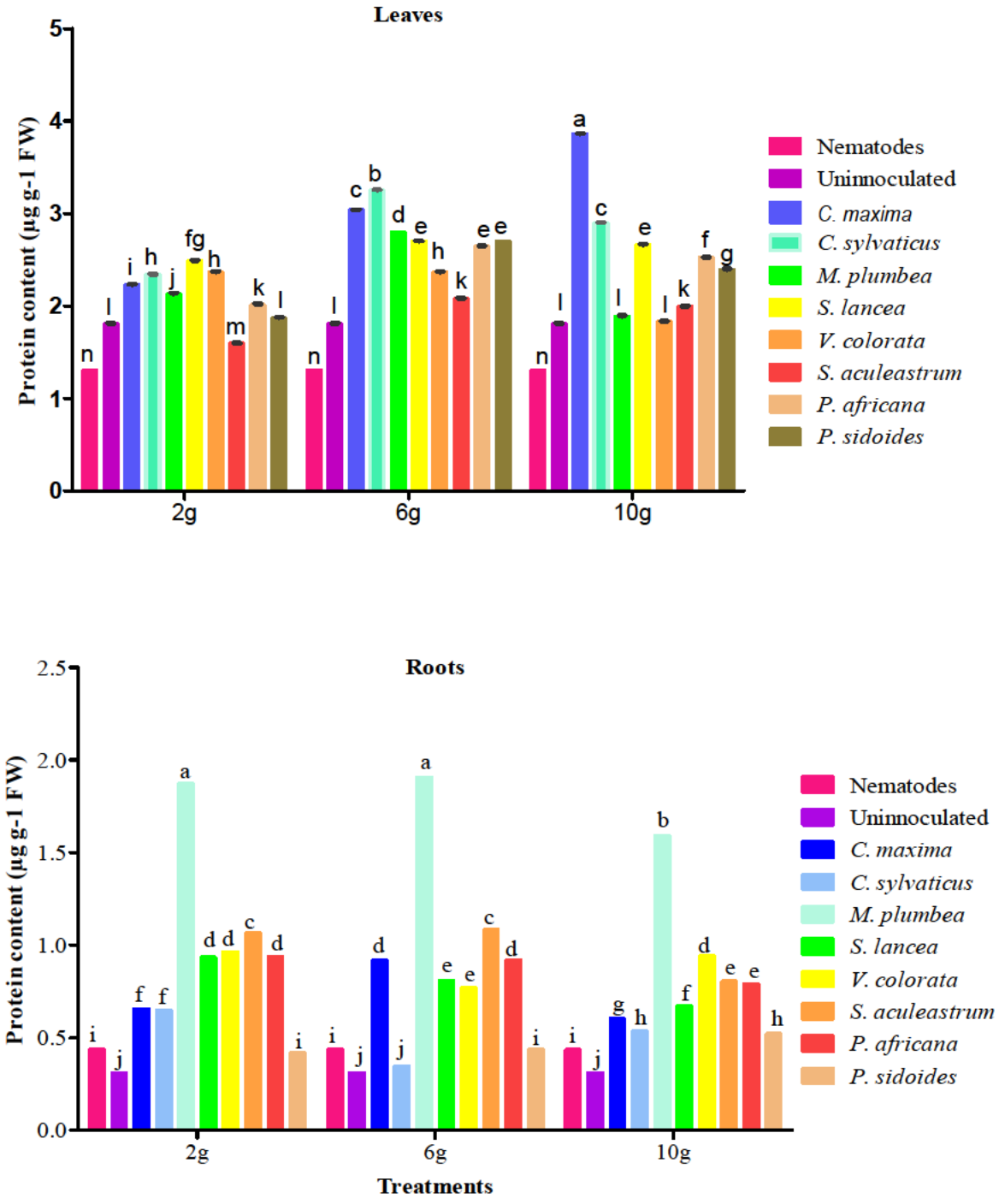

Figure 5.7. Effect of powdered plant material at three application rates on the total protein content of tomato seedlings infected with *M. incognita* relative to the untreated and uninoculated control.

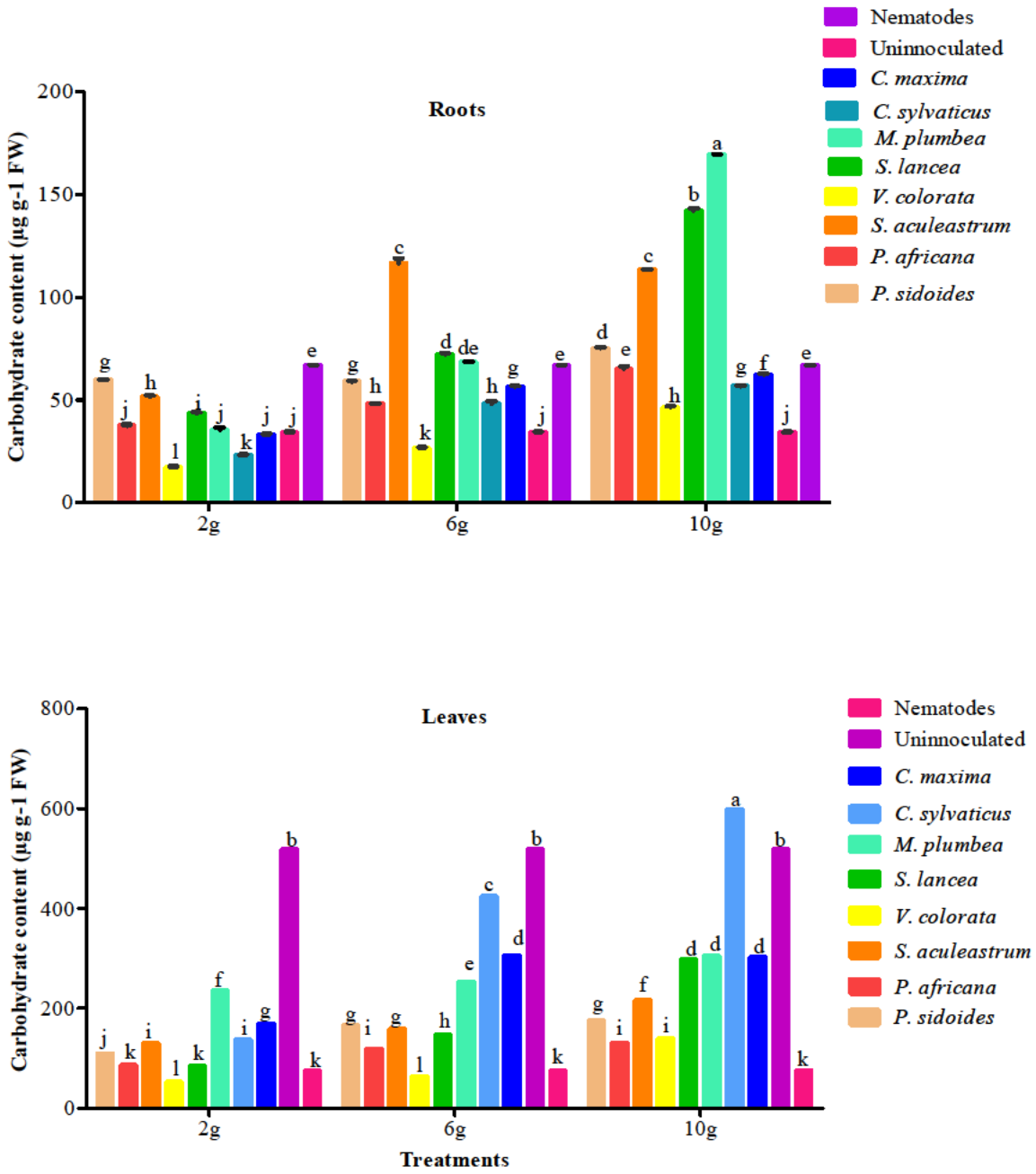

Figure 5.8. Effect of powdered plant material at three application rates on the total carbohydrate contents of tomato seedlings infected with *M. incognita* relative to the untreated and uninoculated control.

Table 5.6. Effect of ground plant material on total phenolic and flavonoids content of tomato-infected with *Meloidogyne incognita*.

Plant species	Trt (g)	Phenolics (mg GAEg^{-1})	Flavonoids (mg CEg^{-1})	Plant species	Trt (g)	Phenolics (mg GAEg^{-1})	Flavonoids (mg CEg^{-1})
P. sidoides	2	270.82±0.31^{b}	1454.8±2.22^{a}	*S. lancea*	2	221.74±0.22^{j}	158.1±0.54^{n}
	6	202.52±0.38^{m}	871.3±0.96^{cd}		6	229.97±0.31^{h}	232.1±0.46^{l}
	10	190.84±0.38^{o}	810.2±2.07^{e}		10	263.61±0.61^{c}	902.2±1.04^{c}
P. africana	2	235.47±0.23^{g}	846.6±0.42^{cd}	*M. plumbea*	2	149.66±0.33^{s}	582.7±1.11^{i}
	6	289.36±0.31^{a}	456.8±1.50^{j}		6	204.57±0.23^{lm}	558.4±1.49^{i}
	10	183.98±0.23^{p}	184.0±0.42^{mn}		10	229.97±0.33^{h}	646.7±0.55^{gh}
S. aculeastrum	2	248.85±0.23^{e}	172.7±1.17^{mn}	*C. sylvaticus*	2	224.14±0.33^{i}	211.6±0.36^{lm}
	6	261.21±0.23^{d}	193.3l±0.84^{m}		6	204.92±0.31^{l}	629.5±0.96^{gh}
	10	195.65±0.31^{n}	363.9±0.34^{k}		10	114.99±0.23^{u}	1069.1±2.13^{b}
V. colorata	2	244.39±0.38^{f}	355.1±0.70^{k}	*C. maxima*	2	182.95±0.23^{pq}	701.9±1.80^{f}
	6	176.43±0.23^{r}	236.3±0.63^{l}		6	212.13±0.31^{k}	669.7±1.31^{gh}
	10	135.84±0.31^{t}	376.9±0.72^{k}		10	243.70±0.55^{f}	638.5±1.87^{gh}
Uninoculated	Water	180.89±0.23^{q}	660.5±0.08^{gh}	Inoculated	water	192.90±0.23^{o}	708.8±1.01^{f}

Means are averages of five replicates. Column means followed by the same letter(s) are not significantly different at $p \leq 0.05$ according to the Waller-Duncan Multiple Range test.

Table 5.7. Effect of ground plant material on nematodes juvenile (J2s) densities and eggs on root systems of *Meloidogyne incognita* on tomato plants at 56-days after inoculation with 2 000 J2s and eggs and application of treatment.

Plant species	Trt (g)	Eggs	J2s	Plant species	Trt (g)	Eggs	J2s
P. sidoides	2	856^{c-e}	25872^{b}	*S. lancea*	2	768^{c-g}	14472^{e-i}
	6	840^{c-f}	21640^{b-d}		6	648^{e-h}	9936^{h-n}
	10	608^{e-h}	13440^{f-k}		10	384^{g-i}	7640^{l-o}
P. africana	2	1112^{cd}	17480^{d-f}	*M. plumbea*	2	768^{c-g}	23856^{bc}
	6	920^{c-e}	14896^{e-i}		6	360^{g-i}	15440^{e-h}
	10	848^{c-e}	13888^{f-j}		10	304^{hi}	8024^{k-o}
S. aculeastrum	2	1144^{c}	19872^{c-e}	*C. sylvaticus*	2	688^{d-h}	9880^{i-n}
	6	616^{e-h}	16032^{e-g}		6	664^{e-h}	6768^{m-o}
	10	416^{f-i}	13120^{f-l}		10	660^{e-h}	6184^{m-p}
V. colorata	2	528^{e-i}	8856^{j-n}	*C. maxima*	2	1792^{b}	11096^{g-m}
	6	264^{hi}	5064^{n-p}		6	1760^{b}	9864^{i-n}
	10	112^{i}	3184^{op}		10	688^{d-h}	896^{p}
Control	Water	14728^{a}	61208^{a}				

Means are averages of five replicates. Column means followed by the same letter(s) are not significantly different at $p \leq 0.05$ according to the Waller-Duncan Multiple Range test.

5.3.3. Experiment 3

5.3.3.1. Effect of botanicals and PGPR on nematode-infected tomato plant growth parameters

Treatments had a significant (p<0.05) effect on shoot height (**Figure 5.9, 5.10, 5.11 and 5.13**), stem diameter, root length (**Figure 5.9 to 5.12**), fresh shoot weight, fresh root weight and dry shoot weight (**Appendix 5.4**), with co-treatments contributing 25, 15, 6, 14, 10 and 3%, respectively in TTV, while bacterial strains alone contributed 23, 46, 77, 8, 42 and 68%, respectively in TTV. Relative to the untreated control, plant shoot length was increased by the application of co-treatment (**Table 5.8a and b**). The most significant improvement was produced by applying *C. sylvaticus*+*Bl*, *P. sidoides*+*Bl*, *C. maxima*+*Bl,* and *M. plumbea*+*Bl*. A minor improvement was noted on shoot length of plants treated with *P. africana*+*Bl* and *S. lancea*+*Bl*. However, *Bl* applied alone significantly reduced shoot length by 29%, while *Pf* enhanced shoot length by 39%, but it was still lower than the uninoculated plants. *S lancea* combined with either *Bl* or *Pf* reduced root length by 25% and 12%, respectively (**Table 5.8a and b**). Plants treated with botanicals mixed with *Bl* showed the greatest increase in root length (41 to 185%), while plants treated with botanicals combined with *Pf* showed a minor decrease (4 to 43%). However, tomato plants treated with *Bl* alone produced an increase in root length by 52%, while *Pf* alone recorded 4% enhancement.

Tomato plants treated with *C. maxima*, *C. sylvaticus*, *M. plumbea* and *S. aculeastrum* combined with *Bl* or *Pf* showed higher significant improvement on fresh shoot weight respectively increased by 193, 252, 178, and 95% and 185, 197, 87, and 84%. Also, the same co-treatments enhanced dry shoot weight by 160, 213, 105 and 43%, and 250, 250, 173 and 115%,

respectively. *Cucurbita maxima* and *C. sylvaticus* combined with *Bl* or *Pf* exhibited a stimulatory effect on fresh and dry root weight with a significant improvement of 163% and 241%, and 418% and 624%, respectively. Plants treated with *Bl* alone reduced dry root weight by 37%, while *Pf* increased dry root weight by 89% (**Table 5.8a and b**). When compared to botanicals combined with *Bl*, all tomato plants treated with botanicals combined with *Pf* improved plant stem diameter. When compared to untreated plants, *Bl* and *Pf* alone increased stem diameter by 1% and 21%, respectively. (**Table 5.8c**).

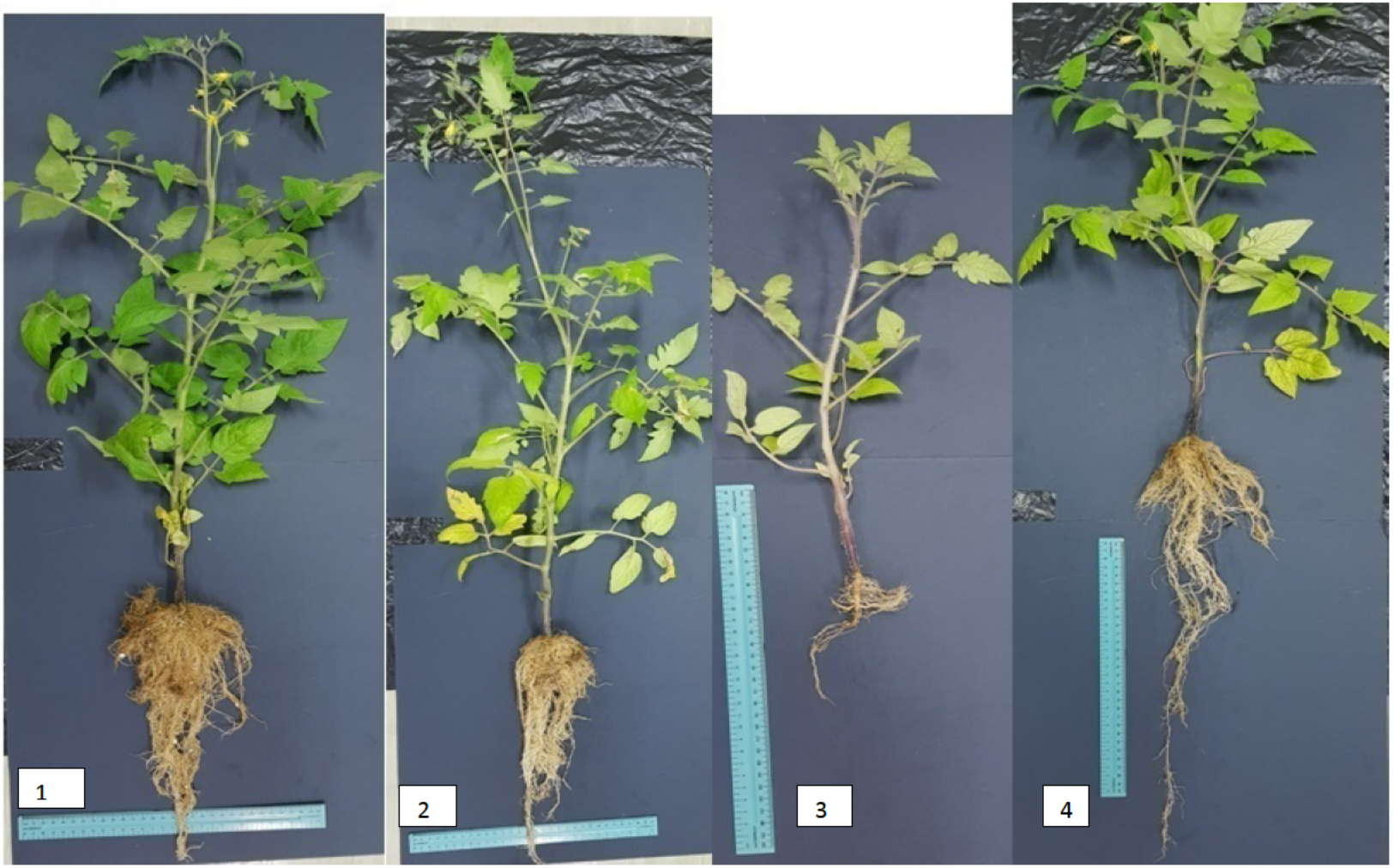

Figure 5.9. Effect of treatments on plant growth of nematode-infected tomato seedlings.1 = *C. sylvaticus*+*Bl*, 2 = *M. plumbea*+*Bl*, 3 = *S. lancea*+*Bl*, 4 = *V. colorata*+*Bl*.

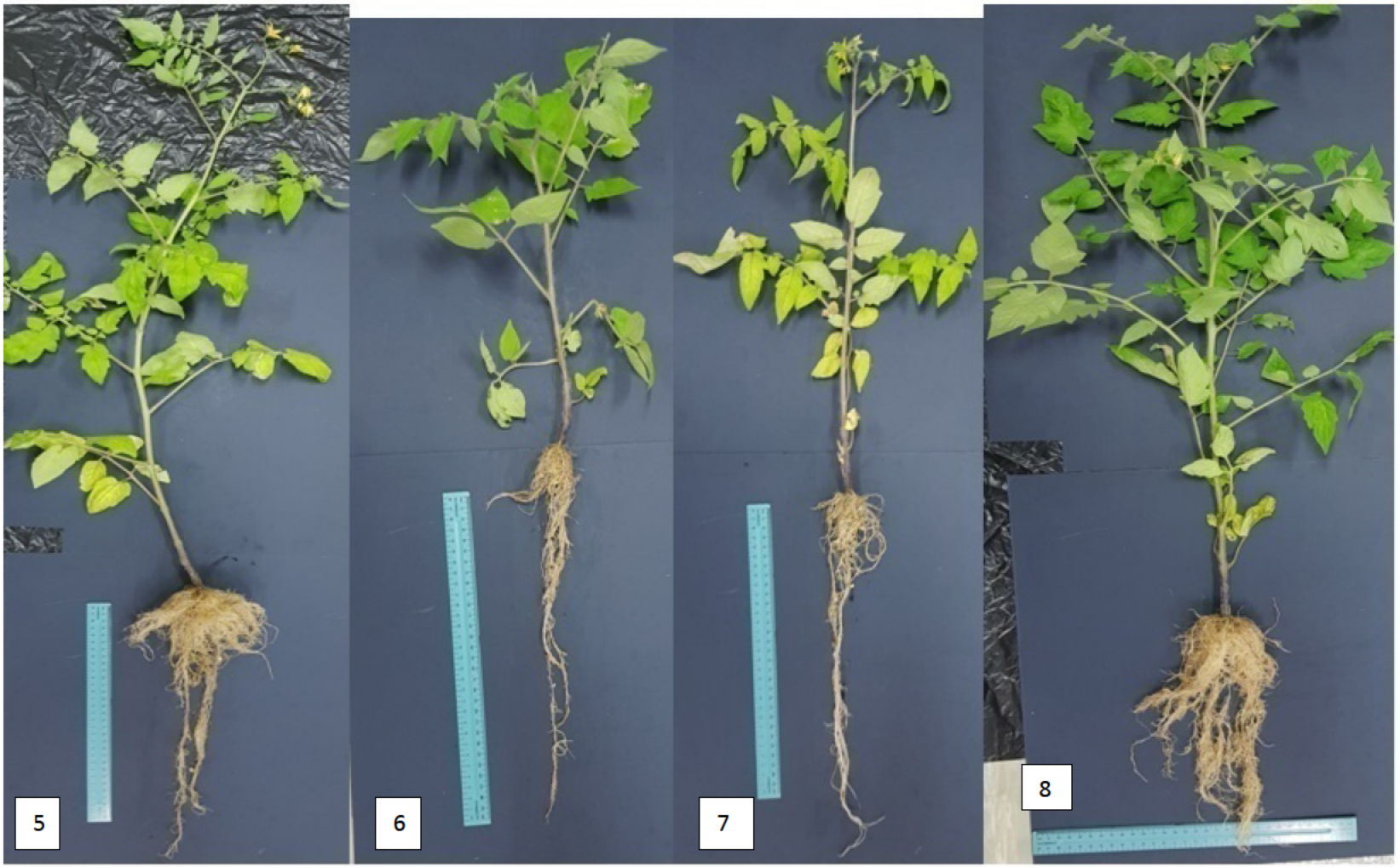

Figure 5.10. Effect of treatments on plant growth of nematode-infected tomato seedlings. 5 = *S. aculeastrum+Bl*, 6 = *P. africana+Bl*, 7 = *P. sidoides+Bl* and 8 = *C. maxima+Bl*.

Figure 5.11. Effect of treatments on plant growth of nematode-infected tomato seedlings. 9 = *Bl* alone.

Figure 5.12. Effect of treatments on root gall formation on nematode-infected tomato seedlings when compared to the untreated control. A = roots of untreated control showing severe infection by the root-knot nematode with visible root galls and egg masses; B = show roots of treated (*C. maxima* + *Pf*) tomato roots with tiny root galls and no egg masses.

Figure 5.13. Effect of treatments (plant powdered material combined with *Pf*) on plant growth of nematode-infected tomato seedlings. From the right side: *P. sidoides*+*Pf*, *P. africana*+*Pf*, *S. aculeastrum*+*Pf*, *V. colorata*+*Pf*, *S. lancea*+*Pf*, *M. plumbea*+*Pf*, *C. sylvaticus*+*Pf*, *C. maxima*+*Pf*, *Pf* alone and untreated nematode free control.

5.3.3.2. Effect of botanicals and PGPR on photosynthetic pigments of the nematode-infected tomato plant

Photosynthetic pigments such as chlorophyll a, b, and a+b and carotenoid content were evaluated (**Table 5.9**). The chlorophyll content decreased in the untreated plants compared to treated plants (**Table 5.9**). Treated plants with botanicals combined with *Bl* showed the highest improvement in chlorophyll a+b content, with *S. lancea*+*Bl* being the least effective treatment. In comparison to the untreated control, chlorophyll a+b content was slightly decreased by applying botanicals combined with *Pf* except for those treated with *S. lancea*+*Pf* and *M. plumbea*+*Pf.* Moreover, plants treated with botanicals combined with *Pf* significantly improved chlorophyll b content compared to plants treated with botanicals combined with *Bl*, except for plants treated with *P. africana, V. colorata, C sylvaticus* and *C. maxima*. It was also determined that untreated plants had lower carotenoid concentration than uninoculated plants. When compared to uninoculated plants, the application of *S. lancea*+*Bl* and *Bl* alone considerably reduced carotenoid content, however only *P. africana*+*Pf* and *V. colorata*+*Pf* generated similar reduction trends.

5.3.3.3. Effect of botanicals and PGPR on the estimated total carbohydrate and protein content of the nematode-infected tomato plant

Nematode infection led to increased total carbohydrate and protein contents of tomato seedlings (**Table 5.10**). The protein content in roots of tomato was significantly lower in plants treated with botanicals combined with *Bl* while botanicals+*Pf* increased protein content. Similar trends were noted in the carbohydrate content. Protein in leaves was significantly reduced in the untreated nematode-free plants. The highest increase was observed in plants treated with *V. colorata*+*Pf*. Relative to the untreated control; the tested treatments had significant lower levels

of carbohydrate in either the roots or the leaves. However, *P. africana+Pf* had the highest leaf carbohydrate content, and *V. colorata+Pf* had the highest root carbohydrate content.

5.3.3.4. Effect of botanicals and PGPR on the estimated flavonoids and total phenolic content of the nematode-infected tomato plant

Relative to the uninoculated tomato plants, the levels of flavonoid and total phenolic contents increased in the untreated control by 130 and 19%, respectively (**Table 5.11**). Furthermore, infected plants treated with *V. colorata+Bl* and *C. sylvaticus+Bl* led to further increases in the total phenolic content by 4 and 8%, respectively. The treatment of the infected plants with *S. aculeastrum+Bl*, *P. sidoides+Bl*, *P.africana+Bl*, *P. sidoides+Pf* and *V. colorata+Pf* increased the levels of flavonoids by 101, 51, 2, 35 and 54%, respectively.

5.3.3.5. Effect of botanicals and PGPR on nematode densities.

Treatments had a significant effect on number of juveniles in soil, number of eggs and juveniles in roots and the reproduction factor, with co-treatments contributing 3, 17, 5 and 1%, respectively, in TTV of the four variables (**Appendix 5.5**), whereas bacterial strains alone significantly affected juveniles in soil, number of eggs and juveniles in roots and the reproduction factor, contributing 95, 57, 81 and 98%, respectively, in TTV of the four variables. Significantly (p<0.05), all treatments reduced juvenile numbers and egg numbers in roots, while juvenile numbers in soil were stimulated only in potted soil treated with *S. lancea*+Pf, *M. plumbea+Pf* and *Pf* (**Table 5.12**). The final nematode population densities were significantly suppressed in plants treated with *C. maxima+Bl* with an RF value of 0.30. Therefore, the results

of this current study suggest that treatments were able to inhibit juvenile penetration and resulted in reduced root gall formation. A similar result was observed in **Chapter 4**.

Table 5.8a. Effect of *B. licheniformis* (*Bl*) with botanicals on plant growth parameters of nematode-infested tomato plants.

	Length (cm)				Weight (g)							
	Root		Shoot		Fresh roots		Dry roots		Fresh shoot		Dry shoot	
Treatment	Mean	%	Mean	%	Mean	%	Mean	%	Mean	%	Mean	%
P. sidoides+Bl	39.77±0.66^{b}	106	70.93±7.79^{ab}	59	9.09±3.03^{cd}	-7	1.29±1.76^{b}	65	35.75±12.80^{bc}	142	4.30±1.73^{bc}	87
P. africana+Bl	27.37±4.45^{cde}	41	38.90±6.26^{de}	-13	3.07±0.73^{e}	-66	0.36±0.08^{c}	-54	12.02±3.08^{f}	-19	1.29±0.15^{d}	-44
S. aculeastrum+Bl	36.50±4.29^{bc}	89	62.57±12.72^{abc}	40	11.65±0.63^{c}	19	1.42±0.07^{b}	82	28.85±5.98^{d}	95	3.28±1.06^{c}	43
V. colorata+Bl	55.17±2.60^{a}	185	54.20±6.55^{bcd}	22	12.37±1.06^{c}	27	1.43±0.04^{b}	83	19.74±6.11^{e}	34	1.62±1.13^{d}	-30
S. lancea+BL	14.57±4.83^{f}	-25	38.57±4.85^{de}	-14	1.39±0.34^{e}	-86	0.23±0.06^{c}	-71	8.34±1.76^{f}	-44	0.81±0.22^{d}	-65
M. plumbea+ Bl	34.23±2.17^{bcd}	77	70.73±8.47^{ab}	59	13.50±1.68^{c}	38	1.43±0.05^{b}	83	41.04±8.90^{ab}	178	4.71±1.57^{abc}	105
C. sylvaticus+Bl	35.70±1.94^{bcd}	85	77.93±1.41^{a}	75	33.30±3.57^{a}	241	5.65±0.43^{a}	624	52.09±5.04^{a}	252	7.19±0.74^{a}	213
C. maxima+Bl	32.20±1.05^{bcd}	66	74.33±0.27^{a}	68	25.75±1.81^{b}	163	4.04±0.65^{a}	418	43.31±1.08^{ab}	193	5.98±0.16^{ab}	160
B. licheniformis	29.30±7.67^{de}	52	31.53±7.07^{f}	-29	5.41±2.47^{d}	-44	0.49±0.22^{c}	-37	8.20±1.88^{e}	-44	1.26±0.01^{d}	-45
Untreated	19.35±0.08^{f}	-	44.57±0.44^{d}	-	9.78±0.12c	-	0.78±0.04c	-	14.76±0.20^{d}	-	2.30±0.15^{cd}	-
Uninoculated	26.47±0.20^{e}	37	55.21±0.92^{bcd}	24	14.49±0.48^{d}	48	1.49±0.0^{d}	91	44.30±1.74^{ab}	200	8.42±0.01^{a}	266

[Y]Means ± SE are averages of six replicates. Column means followed by the same letter(s) are not significantly different at $p \leq 0.05$ according to the Waller-Duncan Multiple Range test. [z]Impact (%) = $\left(\frac{\text{treatment}}{\text{control}} - 1\right) * 100$. Percentage values with a negative sign show a decrease in plant growth parameters, while those without a negative sign indicate an increase in the measured parameter.

Table 5.8b. Effect of *P. fluorescens* (*Pf*) with botanicals on plant growth parameters of nematode-infested tomato plants.

	Length (cm)				Weight (g)							
	Root		Shoot		Fresh roots		Dry roots		Fresh shoot		Dry shoot	
Treatment	Mean	%	Mean	%	Mean	%	Mean	%	Mean	%	Mean	%
P. sidoides+Pf	26.01±0.42^{ab}	34	62.92±0.32^{bc}	41	15.93±0.31^{cde}	63	1.34±0.10^{c}	72	31.94±0.37^{b}	116	5.99±0.16^{b}	160
P. africana+Pf	20.17±0.14^{cd}	4	67.58±0.26^{ab}	52	8.96±0.47^{g}	-8	0.79±0.08^{d}	1	23.85±0.33^{cd}	62	4.11±0.15^{c}	79
S. aculeastrum+Pf	26.44±0.26^{ab}	37	68.72±0.29^{a}	54	18.12±0.29^{bc}	85	1.53±0.06^{b}	96	27.18±0.26^{c}	84	4.95±0.13^{bc}	115
V. colorata+Pf	24.86±0.25^{ab}	29	66.28±0.25^{ab}	49	14.23±0.46d^{ef}	46	1.20±0.11^{c}	54	24.61±0.37^{cd}	67	4.05±0.18^{c}	76
S. lancea+Pf	17.03±0.14^{d}	-12	55.90±0.14^{de}	25	11.57±0.24^{fg}	18	0.98±0.09^{d}	26	25.15±0.33^{cd}	70	5.12±0.24^{b}	123
M. plumbea+Pf	27.64±0.37^{a}	43	55.56±0.37^{de}	25	16.14±0.19^{cd}	69	1.36±0.07^{c}	74	27.56±0.44^{c}	87	6.28±0.11^{b}	173
C. sylvaticus+Pf	23.63±0.13^{bc}	22	64.38±0.35^{abc}	44	29.82±0.37^{b}	205	1.76±0.11^{b}	125	43.84±0.25^{a}	197	8.06±0.26^{a}	250
C. maxima+Pf	25.63±0.11^{ab}	33	60.40±0.30^{cb}	36	31.35±0.18^{a}	221	2.64±0.08^{a}	239	42.09±0.31^{a}	185	8.60±0.29^{a}	250
P. fluorescens	20.13±0.19^{cb}	4	49.42±0.56^{f}	11	13.08±0.27^{ef}	34	1.47±0.08^{b}	89	24.62±0.29^{cd}	67	3.73±0.13^{c}	62
Untreated	19.35±0.08^{d}	-	44.57±0.44^{ef}	-	9.78±0.12^{c}	-	0.78±0.04^{d}	-	14.76±0.20^{d}	-	2.30±0.15^{c}	-
Uninoculated	26.47±0.20^{ab}	38	55.21±0.92^{e}	24	14.49±0.48^{d}	48	1.49±0.0^{b}	91	44.30±1.74^{a}	200	8.42±0.01^{d}	266

[Y]Means ± SE are averages of six replicates. Column means followed by the same letter(s) are not significantly different at $p \leq 0.05$ according to the Waller-Duncan Multiple Range test. [z]Impact (%) = $\left(\frac{\text{treatment}}{\text{control}} - 1\right) * 100$. Percentage values with a negative sign show a decrease in plant growth parameters, while those without a negative sign indicate an increase in the measured parameter.

Table 5.8c. Effect of *B. licheniformis* and *P. fluorescens* with botanicals on plant growth parameters of nematode-infested tomato plants.

Treatments	*Bacillus licheniformis*				*Pseudomonas fluorescens*			
	Stem diameter		Root gall index		Stem diameter		Root gall index	
	Mean	%	Mean	%	Mean	%	Mean	%
P. sidoides	6.56±0.21^{abc}	30	3^{c}	-40	6.74±0.07^{abc}	34	4^{b}	-20
P. africana	5.18±0.43^{bcde}	3	3^{c}	-40	5.94±0.26^{bcde}	18	3^{c}	-40
S. aculeastrum	5.94±0.03^{abcde}	18	3^{c}	-40	6.73±0.29^{ab}	34	3^{c}	-40
V. colorata	4.72±0.16^{de}	-6	2^{d}	-60	6.51±0.25^{abc}	29	3^{c}	-40
S. lancea	4.66±0.08^{e}	-8	4^{b}	-20	6.17±0.14^{abcd}	22	3^{c}	-40
M. plumbea	6.95±0.07^{ab}	38	3^{c}	-40	5.64±0.37^{abcde}	12	4^{b}	-20
C. sylvaticus	7.38±0.19^{a}	46	3^{c}	-40	6.63±0.13^{ab}	32	4^{b}	-20
C. maxima	6.45±0.34^{abcd}	28	3^{c}	-40	7.32±0.11^{a}	45	3^{c}	-40
Bacteria	5.08±0.17^{cde}	1	5^{a}	0	6.10±0.19^{abcd}	21	5^{a}	0
Untreated	5.04±0.06^{cde}	-	5^{a}	-	5.04±0.06^{cde}	-	5^{a}	-
Uninoculated	5.41±0.03^{bcde}	7	-	-	5.41±0.03^{bcde}	7	-	-

[Y]Means ± SE are averages of six replicates. Column means followed by the same letter(s) are not significantly different at $p \leq 0.05$ according to the Waller-Duncan Multiple Range test. [z]Impact (%) = $\left(\frac{\text{treatment}}{\text{control}} - 1\right) * 100$. Percentage values with a negative sign show a decrease in plant growth parameters, while those without a negative sign indicate an increase in the measured parameter.

Table 5.9. Effect of *B. licheniformis* and *P. fluorescens* with botanicals on photosynthetic pigmentation of nematode-infected tomato plants.

Treatments	*Bacillus licheniformis*				*Pseudomonas fluorescens*			
	Chlorophyll a µg g^{-1} FW	**Chlorophyll b µg g^{-1} FW**	**Chlorophyll a+b µg g^{-1} FW**	**Carotenoid µg g^{-1} FW**	**Chlorophyll a µg g^{-1} FW**	**Chlorophyll b µg g^{-1} FW**	**Chlorophyll a+b µg g^{-1} FW**	**Carotenoid µg g^{-1} FW**
P. sidoides	343 ± 12.2^{d}	111 ± 2.5^{f}	454 ± 9.7^{f}	99 ± 1.5^{e}	326.14±1.0^{b}	184.07±1.4^{b}	510.21±2.4^{b}	114.66±0.2^{d}
P. africana	471 ± 0.07^{b}	151 ± 0.7^{c}	622 ± 0.6^{c}	125 ± 0.2^{c}	225.62±0.1^{c}	130,13±0.8^{d}	355.74±0.7^{d}	98.11±0.1^{e}
S. aculeastrum	363±0.4^{cd}	121 ± 0.3^{ef}	484 ± 0.1^{e}	95 ± 4.3^{e}	376.76±0.7^{b}	181.86±0.8^{b}	558.62±1.4^{b}	136.46±0.3^{c}
V. colorata	396±1.9^{c}	131 ± 2.0^{de}	527 ± 3.9^{d}	109 ± 0.6^{d}	226.81±1.9^{c}	86.29±0.0^{e}	313.10±0^{d}	93.65±0.2^{e}
S. lancea	266±0.8^{f}	77 ± 0.3^{g}	343 ± 0.9^{g}	76 ± 0.19^{f}	433.66±0.8^{a}	179.63±0.9^{b}	613.29±1.3^{a}	168.29±0.4^{a}
M. plumbea	363±39.8^{cd}	145±14.7^{cd}	508 ± 25de	116 ± 3.8^{d}	444.61±0.1^{a}	165.48±0.1^{c}	610.09±1.3^{a}	165.36±0.4^{a}
C. sylvaticus	531 ± 2.1^{a}	200 ± 0.4^{a}	732 ± 1.7^{a}	156 ± 5.1^{a}	371.36±0.4^{b}	158.31±0.3^{c}	529.67±0.9^{b}	146.63±0.0^{b}
C. maxima	504 ± 1.7^{ab}	181 ± 10.1^{b}	685±8.4^{b}	141 ± 3.2^{b}	341.77±0.4^{b}	168.56±0.3^{c}	510.33±0.9^{b}	133.12±0.1^{c}
Bacteria	228 ± 0.2^{f}	89 ± 0.2^{g}	318±0.03^{gh}	79 ± 0.1^{f}	415.41±0.6^{a}	191.08±0.6^{a}	606.50±0.9^{a}	142.64±0.2^{b}
Uninoculated	306.00±0.3^{e}	154.39±0.7^{c}	460.39±0.5^{f}	112.85±0.1^{d}	306.00±0.3^{b}	154.39±0.7^{c}	460.39±0.5^{c}	112.85±0.1^{d}
Untreated	169 ± 0.4^{g}	58 ± 0.0^{h}	227 ± 0.4^{i}	62 ± 0.1^{g}	169 ± 0.4^{d}	58 ± 0.0^{f}	227±0.4^{e}	62 ± 0.1^{f}

[Y]Means ± SE are averages of six replicates. Column means followed by the same letter(s) are not significantly different at $p \leq 0.05$ according to the Waller-Duncan Multiple Range test.

Table 5.10. Effect of *B. licheniformis* and *P. fluorescens* with botanicals on protein and carbohydrate contents of nematode-infected tomato plants.

	Bacillus licheniformis				***Pseudomonas fluorescens***			
Treatments	**Protein leaf µg g^{-1} FW**	**Protein root µg g^{-1} FW**	**Carbohydrate leaf µg g^{-1} FW**	**Carbohydrate root µg g^{-1} FW**	**Protein leaf µg g^{-1} FW**	**Protein root µg g^{-1} FW**	**Carbohydrate leaf µg g^{-1} FW**	**Carbohydrate root µg g^{-1} FW**
P. sidoides	16.8 ± 0.07^{f}	2.3 ± 0.05^{h}	166.5 ± 0.46^{d}	16.1 ± 0.15^{b}	33.01 ± 0.08^{c}	15.92 ± 0.08^{g}	408.45 ± 0.11^{g}	46.07 ± 0.11^{j}
P. africana	20.9 ± 0.07^{ce}	3.9 ± 0.05^{f}	87.0 ± 0.23^{k}	7.0 ± 0.23^{h}	32.87 ± 0.08^{cd}	32.44 ± 0.33^{e}	645.70 ± 0.20^{a}	86.99 ± 0.11^{d}
S. aculeastrum	21.8 ± 0.03^{cde}	2.9 ± 0.06^{g}	154.8 ± 0.15^{e}	11.3 ± 0.15^{f}	32.99 ± 0.05^{c}	28.88 ± 0.34^{f}	607.04 ± 0.15^{b}	93.68 ± 0.07^{c}
V. colorata	23.2 ± 2.42^{e}	4.8 ± 0.03^{c}	93.6 ± 0.62^{h}	8.9 ± 0.09^{g}	49.33 ± 0.05^{a}	49.07 ± 0.35^{d}	591.23 ± 0.13^{c}	96.38 ± 0.14^{b}
S. lancea	17.4 ± 0.05^{f}	6.6 ± 0.06^{b}	88.7 ± 0.27^{j}	7.6 ± 0.09^{h}	32.32 ± 0.05^{d}	60.94 ± 0.13^{b}	492.82 ± 0.13^{e}	117.71 ± 0.07^{a}
M. plumbea	27.6 ± 0.06^{b}	4.1 ± 0.03^{e}	168.9 ± 0.15^{c}	12.6 ± 0.00^{e}	29.17 ± 0.024^{e}	48.67 ± 0.25^{d}	242.46 ± 0.07^{i}	63.56 ± 0.10^{g}
C. sylvaticus	28.9 ± 0.05^{ab}	4.6 ± 0.05^{d}	284.2 ± 0.32^{a}	7.0 ± 0.09^{h}	22.96 ± 0.06^{f}	52.08 ± 0.14^{c}	377.25 ± 0.18^{h}	54.87 ± 0.11^{h}
C. maxima	30.2 ± 0.10^{a}	6.5 ± 0.03^{b}	126.2 ± 0.46^{f}	36.6 ± 0.31^{a}	19.96 ± 0.08^{g}	51.44 ± 0.18^{cd}	233.50 ± 0.19^{j}	72.47 ± 0.07^{e}
Bacteria	23.2 ± 0.03^{cd}	9.8 ± 0.07^{a}	102.7 ± 0.23^{g}	15.3 ± 0.15^{c}	29.17 ± 0.07^{e}	49.74 ± 0.34^{d}	475.28 ± 0.14^{f}	48.39 ± 0.07^{i}
Untreated	38.32 ± 0.08^{b}	72.21 ± 0.17^{a}	502.54 ± 0.18^{d}	68.96 ± 0.11^{c}	38.32 ± 0.08^{b}	72.21 ± 0.17^{a}	502.54 ± 0.18^{d}	68.96 ± 0.11^{c}
No inoculations	17.5 ± 0.03^{f}	2.0 ± 0.06^{i}	246.3 ± 0.15^{b}	14.60 ± 0.39^{d}	17.5 ± 0.03^{h}	2.0 ± 0.06^{h}	246.3 ± 0.15^{i}	14.60 ± 0.39^{k}

[Y]Means ± SE are averages of six replicates. Column means followed by the same letter(s) are not significantly different at $p \leq 0.05$ according to the Waller-Duncan Multiple Range test.

Table 5.11. Effect of *B. licheniformis* and *P. fluorescens* with botanicals on total phenolic and flavonoid contents of nematode-infected tomato plants.

Treatments	*Bacillus licheniformis*		*Pseudomonas fluorescens*	
	Phenolics (mg GAEg^{-1})	**Flavonoids (mg CEg^{-1})**	**Phenolics (mg GAEg^{-1})**	**Flavonoids (mg CEg^{-1})**
P. sidoides	197.37±0.23^{j}	831.12±1.53^{b}	155.15± 0.23^{o}	748.31±0.76^{c}
P. africana	261.90±0.21^{e}	560.90±0.89^{d}	241.99± 0.07^{f}	478.52±1.61^{e}
S. aculeastrum	236.84±0.23^{g}	1108.95±1.54^{a}	128.37± 0.33^{p}	292.47±0.83^{hi}
V. colorata	282.49±0.23^{b}	460.11±1.75^{e}	191.53 ± 0.31^{k}	848.33±1.33^{b}
S. lancea	168.88±0.31^{n}	233.05±0.54^{l}	154.12 ± 0.33^{o}	366.01±1.07^{g}
M. plumbea	267.39±0.52^{d}	311.63±0.81^{h}	186.73± 0.38^{l}	231.34±0.49^{l}
C. sylvaticus	292.79±0.33^{a}	406.50±0.59^{f}	180.23± 0.99^{m}	236.71± 0.92^{kl}
C. maxima	216.93±0.46^{i}	304.53±0.49^{jk}	166.13 ± 0.23^{n}	186.11 ± 0.69^{m}
Bacteria	223.45±0.00^{h}	258.56±0.34^{hi}	199.77 ± 0.00^{j}	281.56± 0.00^{ij}
Untreated (inoculated)	271.85±0.08^{c}	550.97±0.17^{d}	271.85±0.0^{c}	550.97±0.17^{d}
Uninoculated	233.06±0.03^{g}	239.71± 0.06^{hl}	233.06±0.03^{g}	239.71± 0.06^{hl}

[Y]Means ± SE are averages of six replicates. Column means followed by the same letter(s) are not significantly different at $p \leq 0.05$ according to the Waller-Duncan Multiple Range test.

Table 5.12. Effect of *B. licheniformis* and *P. fluorescens* with botanicals on *M. incognita* of nematode-infected tomato plants.

	Bacillus licheniformis							***Pseudomonas fluorescens***						
	J2s (Roots)		**Eggs**		**J2s (Soil)**		**Rf**	**J2s (Roots)**		**eggs**		**J2s (Soil)**		**Rf**
Treatments	**Mean**	**%**	**Mean**	**%**	**Mean**	**%**	**Mean**	**Mean**	**%**	**Mean**	**%**	**Mean**	**%**	**mean**
P. sidoides+	287^{d}	-92	20^{d}	-98	6667^{cd}	-73	3.49^{d}	1556^{c}	-54	196^{b}	-84	19450^{bc}	-19	10.60^{b}
P. africana+	600^{b}	-82	33^{d}	-97	9917^{b}	-60	5.28^{c}	2136^{b}	-36	0^{b}	-100	16650^{c}	-33	9.39^{c}
S. aculeastrum	260^{d}	-92	47^{d}	-96	5333^{d}	-79	2.82^{de}	340^{de}	-90	0^{b}	-100	23350^{ab}	-6	11.88^{b}
V. colorata+	167^{de}	-95	0^{d}	-100	7333^{c}	-71	3.75^{e}	764^{d}	-77	0^{b}	-100	10750^{d}	-60	5.76^{c}
S. lancea+	440^{bc}	-87	220^{c}	-82	15500^{a}	-38	8.08^{b}	384^{de}	-89	0^{b}	-100	25000^{a}	0.2	12.69^{a}
M. plumbea+	193^{de}	-94	60^{d}	-95	4500^{d}	-82	2.38^{de}	204^{e}	-94	0^{b}	-100	35600^{a}	43	17.90^{a}
C. sylvaticus	173^{de}	-95	427^{b}	-66	2833^{e}	-89	1.72^{ef}	704^{d}	-79	0^{b}	-100	18800^{bc}	-25	9.38^{c}
C. maxima+	80^{e}	-98	20^{d}	-98	500^{f}	-98	0.30^{g}	160^{e}	-95	0^{b}	-100	20000^{ab}	-20	14.33^{a}
Bacteria	667^{b}	-80	20^{d}	-98	2083^{e}	-92	1.39^{f}	2352^{b}	-30	0^{b}	-100	29550^{a}	18	16.38^{a}
Untreated	3360^{a}	-	1247^{a}	-	24950^{a}	-	15.23^{a}	3360^{a}	-	1247^{a}	-	24950^{a}	-	15.23^{a}

[Y]Means ± SE are averages of six replicates. Column means followed by the same letter(s) are not significantly different at $p \leq 0.05$ according to the Waller-Duncan Multiple Range test. [z]Impact (%) = $\left(\frac{\text{treatment}}{\text{control}} - 1\right) * 100$.Percentage values with a negative sign show a decrease in plant growth parameters, while those without a negative sign indicate an increase in the measured parameter.

5.4. Discussion

Several studies have shown that *Bacillus* and *Pseudomonas* species confer several plant growth-promoting traits; thus, inoculation with PGPR species has been reported to enhance plant growth through direct mechanisms such as phosphorus and iron solubilization from the soil and the production of plant hormones such as indole-3-acetic acid (IAA). In the current study, *Bl* and *Pf* were screened *in vitro* for their plant growth-promoting properties. The production of IAA was observed in both *Bl* and *Pf*. These findings agree with other studies (**Suzuki et al., 2003; Dey et al., 2004; Bharucha et al., 2013; Chen et al., 2017; Wagi and Ahmed, 2019**) who reported IAA production by *Bacillus* and *Pseudomonas* species. Furthermore, *Pf* strain showed the ability to solubilize phosphate compared with *Bl*. Phosphorus is one of the essential micronutrients needed by plants for growth and development, however, phosphorus is found in an insoluble form. Hence, phosphate solubilizing bacteria secrete organic acids and phosphate enzymes which play a role in converting insoluble phosphate into a soluble form.

Moreover, the production of ammonia is a common trait in *Pf* and *Bl* bacteria. Similar results were reported by **Agbodjato et al.** (**2015**), who noted that 80% and 77.77% of *Bacillus* and *Pseudomonas* species, respectively, produced ammonia. Ammonia has been shown to have a variety of impacts on plant-parasitic nematodes, including the mortality of some plant-parasitic nematodes when exposed to low levels of NH_3 (**Oka and Pivonia, 2002**). Furthermore, the results of the current study showed that *Bl* and *Pf* had higher siderophores production capacity. According to **Dey et al.** (**2004**) and **Canbolat et al.** (**2006**) *Pf* and *Bl* have the ability to produce siderophore. **Migunova and Sasanelli (2021**) reported that plant-parasitic nematodes are suppressed by plant growth-promoting rhizobacteria through a variety of processes depending on

the microorganisms' ability to compete for ecological niches, colonize plant root surfaces, and secrete nematicidal substances like siderophores. A positive result for the production of HCN was detected on both *Bacillus* and *Pseudomonas*, while the protease production was only noted as a negative test due to lack of clear halo zone surrounding the colonies. Although, **Brown and Foster** (**1970**) and **Eaton et al.** (**1995**) showed that *Pseudomonas aeruginosa* forms a clear zone around colonies and hydrolyzes casein as evidenced by the production of a yellow to green diffusible pigment, the current findings showed that *Pf* could hydrolyze casein because the yellow pigment was diffused within the medium. In contrast, **Brown and Foster** (**1970**) further reported that the tested *Pf* species could grow on milk agar, but they failed to produce a clear zone, which indicated that any extracellular proteinase produced was not a caseinase. Similarly, in the current study, *Bl* and *Pf* grew on the skimmed milk agar but did not demonstrate any clear zone (**Figure 5.1e**). The ethylene production was barely visible on both bacterial species, while *Pf* showed higher production of NH_3.

The DPPH scavenging radical assay was used in this study to test the free radical scavenging effects of *Pf* and *Bl* extracts. The two bacterial species showed deficient DPPH radical scavenging activity with high IC_{50} values. However, DPPH scavenging activity is primary a method for determining antioxidant activity and should therefore be verified by other more sensitive antioxidant assays. Furthermore, *Pf* showed significantly lower total phenolic and flavonoid contents as compared to *Bl*. The efficacy of bacterial inoculants in the development of crops depends on the complex plant-microbe interaction processes and environmental factors.

Under the greenhouse study, inoculation with *Pf* and *Bl* applied individually significantly reduced plant growth parameters and biochemicals. However, the co-treatment significantly increased plant biomass and physiological properties. Similar results were reported by **Akinrinlola et al.** (**2018**), **Khan et al.** (**2019**), **Ngoroyemoto et al.** (**2020**) and **Bechtaoui et al.** (**2020**).

The application of powdered plant material that is suppressive towards nematode numbers, but has a negative effect on plant growth of the protected host plant, would not be useful as a nematode management alternative. Generally, plant material obtained from various plants has been reported to consist of bioactive compounds that can either stimulate or inhibit plant growth. In this study, the target was plant material that can stimulate plant growth and reduce nematode numbers on tomato plants. The current study showed powdered material of *M. plumbea*, *C. sylvaticus* and *C. maxima* with and without the *Bl* and *Pf* significantly stimulated plant growth and photosynthetic pigmentation of tomato plants (**Experiments 2 and 3**). Furthermore, these three plant species significantly increased both root and shoot weight compared to the untreated tomato plants. Also, the gall index was decreased with the application of these three powdered plant materials in combination with either *Bl* or *Pf*. Similar results have been reported by **Moosavi** (**2012**) when testing six medicinal plant species. **Moosavi** (**2012**) showed that different plants evaluated under the same conditions showed different results. This could be due to the phytochemical properties that plants have, which can either stimulate or inhibit the growth of both plant and nematodes. Hence, **Pelinganga** (**2013**) highlighted that inhibition of plant growth by soil treatments could consequently be due to phytotoxic attributes of the plant material used;

hence **Moosavi et al.** (**2010**) reported that an increase in shoot weight could be a result of better nematode control.

Tomato plants treated with *Bl* nor *Pf* alone had a significant decrease in egg and juvenile numbers. This observation does agree with the previous work of **El-Nagdi et al.** (**2019**), who reported a significant reduction in the numbers of second-stage-juveniles in roots and soil, egg masses, gall index of root-knot nematodes, and increased growth parameters of tomato and eggplant after the application of *B. licheniformis*. Furthermore, our results showed significant effects on plant growth and nematodal parameters in the combined treatments. Opposite findings published by **Mashela and Nthangeni** (**2002**) showed that *Ricinus communis* powdered fruit meal combined with microbes (*Bacillus* species) had no impact on plant growth and *M. incognita* infection rate on tomato plants. However, there is insufficient evidence of powdered plant parts combined with rhizobacteria to control plant pathogenic nematodes. Powdered plant parts and fungal species have stimulatory effects on plant growth factors and inhibitory effects on phytopathogenic nematodes, according to several researches (**Murslain et al., 2014; Feyisa et al., 2015; Niranjana et al., 2018; El-Nagdi et al., 2019**).

The powdered plant materials of *M. plumbea*, *C. maxima, V. colorata, S. aculeastrum* and *C. sylvaticus* significantly reduced *M. incognita* juveniles and egg numbers. The observed nematicidal effects of *M. plumbea*, *C. maxima, V. colorata, S. aculeastrum* and *C. sylvaticus* powdered plant material could be attributed to their richness in secondary metabolites (**Chapter 3; Sithole et al., 2021**). The anthelmintic compounds such as saponins and bufadienolides found in *Merwilla* species (**Sparg et al., 2002**), various fatty acids found in *Cucurbita* species

(**Maldonade et al., 2018**), vernolide and hydroxyvernolides found in *Vernonia* species (**Gasquet et al., 1985**), resin, tannins and alkaloids found in *Solanum* species (**Nfi et al., 1999**) and various compounds found in *Croton* species (**Maroyi**, **2017**) could have played a role in the reduction of nematodes numbers. The results of this study are in agreement with the earlier findings of **Sparg et al.** (**2002**) who reported that *Scilla natalensis* bulbs extract inhibited nematodes' growth and development, **Abolusoro et al.** (**2019**) claimed that *V. amygdaline* leaf powder reduced nematodal population on infested tomato plants, **Hordegen et al.** (**2003**) reported effects of *V. anthelmintica* seed extracts on mixed gastrointestinal nematodes infecting sheep with anthraquinone being the active compound. **Nfi et al.** (**1999**) reported anthelmintic activity of *S. aculeastrum* root extracts on diverse gastrointestinal nematodes infecting bovids, and **Nwude and Ibrahim** (**1980**) reported effects of *S. nodiflorum* fruits on worm infestation. **Ayaz et al.** (**2015**) demonstrated that pumpkin seeds showed anthelmintic activity against nematodes, and **Bodas et al.** (**2014**) reported that nematode paralysis and death caused by *Croton* species were comparable to that caused by the chemical control albendazole, and **Maroyi** (**2017**) reported similar anthelmintic properties of *Croton macrostachyus*.

5.5. Conclusions

Pseudomonas fluorescens (*Pf*) and *Bl* possess plant growth-promoting traits such as phosphate solubilization, iron chelation, and phytohormone production. However, these bacterial strains significantly reduced plant growth as single treatments, but plant growth was increased when the two strains were combined. Such multidimensional utilities of these bacterial strains as co-treatments makes them a bio-agent of choice to be exploited in the field of agriculture. On the other hand, the current study demonstrated that some powdered plant materials could suppress *M. incognita*. It is established that *M. plumbea*, *C. sylvaticus* and *C. maxima* contain

antinematicidal properties that can be of potential in the management of root-knot nematodes as soil amendments. Furthermore, single and co-treatment showed different effects on plant growth variables with *M. plumbea*, *C. sylvaticus* and *C. maxima* combined with either *Bl* or *Pf* exhibiting growth promoting attributes on nematode-infected tomato plants. These results show that these three botanicals combined with either one of the PGPR had a synergistic effect on promoting plant growth and reducing nematode numbers infesting the tomato roots.

CHAPTER 6: Effects of some botanicals on growth and phytochemistry of the nematode-infected *Pelargonium sidoides* and GC-MS profiling of *Cucurbita maxima* seeds

6.1. Introduction

Agriculture continues to be seriously affected by recurrent plant diseases globally, resulting in widespread crop losses and declines in yields (**Savary et al., 2012**). Plant-parasitic nematodes (PPNs) reduce the yields of hundreds of plants, including ornamentals and vegetables that are economically important (**Abd-Elgawad, 2014**). The root-knot nematodes (RKNs) (*Meloidogyne* spp.) are the most destructive polyphagous group of plant pathogens that feed and reproduce on plant roots, leading to gall development (**Mitkowski and Abawi, 2003**), thus, disrupting the critical physiological chemicals of plants. *Pelargonium sidoides* (DC.), commonly referred to as African geranium, is a medicinal herb recommended in the treatment of upper respiratory tract infections. The plant has been widely exploited by local South African traditional healers and international pharmaceutical companies since its discovery in the 1890s (**Bladt and Wagner, 2007**). African geranium is highly susceptible to the RKNs (**Mofokeng et al., 2013; Sithole et al., 2016**) with a decrease in yield quality and quantity.

Chemical nematicides have been used successfully to control RKNs and other PPNs, but their usage is restricted due to health and environmental concerns (**Oka et al., 2000**). In the past few decades, the management of PPNs has shifted towards using natural products as an alternative to chemical nematicides (**Mashela et al., 2007; Renčo et al., 2014**). The use of organic plant material to boost plant growth and increase yields is an old practice that still has excellent traction in agriculture and has been shown to reduce the population densities of some PPNs

significantly (**Mashela et al., 2007; El-Nagdi et al., 2017; El-Nagdi et al., 2019; El-Nagdi and Abd-El-Khair, 2019**). Stimulation of antagonistic microorganisms and leaching of plant secondary volatile/non-volatile chemicals with nematicidal properties through the phenomenon referred to as ground leaching technology (GLT) is part of the process of nematode population reduction by soil amended with organic matter (**Mashela and Nthangeni, 2002**).

Pumpkins are part of the Cucurbitaceae family, which also includes melons, gourds and squashes, and are cultivated globally for their tasty nutritious fruits and seeds. Leaves and stems are consumed as indigenous vegetables in South Africa, although the seeds are normally thrown away. The important Cucurbitaceae species cultivated worldwide are *Cucurbita maxima*, *Cucurbita pepo* and *Cucurbita moschata* (**Lecoq, 2003; Salehi et al., 2019b; Chomicki et al., 2020**). In the last few years, researchers have found that pumpkin seeds contain several important pharmacological qualities (**Dar et al., 2017; El Khatib and Muhieddine, 2019**). The anti-inflammatory, anti-ulcer, anti-diabetic, anti-microbial, and anti-oxidant agents have all been found in pumpkin seed bioactive components (**Yadav et al., 2010; Dar et al., 2017; El Khatib and Muhieddine, 2019**). A study has also shown that pumpkin seeds can be used in the treatment of urinary diseases, as a vermifuge, a preventative against kidney stone formation, and to treat hypertension and benign prostatic hypertrophy (BPH) (**Perez Gutierrez, 2016**). The seed oil contains fatty acids such as palmitic, stearic, oleic, and linoleic acid (**Kulaitiene et al., 2007; Kim et al., 2012**). According to reports, pumpkin seeds offer a wide spectrum of bioactive qualities as well as long-term biological control capability for pathogenic and parasitic organisms, making them useful bioactive chemicals (**Achilonu et al., 2018; Sithole et al., 2021**). However, their impact on root-knot nematodes second-stage juvenile (J2s) remains unreported.

Therefore, the present study aimed to compare the application methods (drenching and mulching) of the eight selected botanicals on the growth of nematode infested *Pelargonium sidoides* under greenhouse conditions and determine the putative bioactive nematicidal compound(s) from *C. maxima* seeds using GC-MS analysis.

6.2. Material and methods

6.2.1. Study location, and plant material collection and preparation

The study was conducted under greenhouse conditions as described previously (**Chapter 5; Section 5.2.1.12**).

Plant material belonging to the eight botanicals viz. *Pelargonium sidoides* (T1), *Prunus africana* (T2), *Solanum aculeastrum* (T3), *Vernonia colorata* (T4), *Searsia lancea* (T5), *Merwilla plumbea* (T6), *Croton sylvaticus* (T7), *Cucurbita maxima* (T8) were collected from the University of KwaZulu-Natal Botanical Garden. The fruit of *C. maxima* was purchased from a local supermarket, and only the seeds were used in this experiment. Plants were identified by the horticulturist, Mrs. Alison Young, at the Department of Life Sciences, University of KwaZulu-Natal, Pietermaritzburg, South Africa. Voucher specimens were deposited at the UKZN, Herbarium (NU), Pietermaritzburg for botanical verification and future reference and prepared as explained previously (**Chapter 4; Section 3.2.1**).

6.2.2. Nematodes inoculation preparation, experimental design and cultural practices

Root-knot nematode (*M. incognita*) inoculum was prepared by extracting eggs and second-stage juveniles (J2s) from roots of the greenhouse-grown nematode-susceptible tomato cv. Roma V.F.

in 1% NaOCl (Sodium hypochlorite) (**Chapter 5; Section 5.2.2.2**). Seeds of *P. sidoides* (African geranium) were purchased from Mountain Herb Estate Nursery, Pretoria, South Africa, and were raised under greenhouse conditions for three months. Eighteen-cm-diameter plastic pots were placed on the greenhouse bench at 0.3 m inter-row and 0.25 m intra-row spacing. Each pot was filled with 2.5 L steam-pasteurised river sand mixed with vermiculite at 2:1 (v/v). Three-month-old *P. sidoides* seedlings were transplanted to these pots. Seven days after transplanting, each pot was inoculated with ±1000 *M. incognita* J2s and eggs using a 50-mL plastic syringe by infesting approximately 10-cm-deep into the soil at cardinal points (around/all directions) of the roots of the plants.

After seven days, 10 g of each ground plant material were separately used to treat *P. sidoides* seedlings infested with nematodes by applying them within a 5-cm-radius around the seedlings, while 5 and 2.5 $mgmL^{-1}$ crude extract was applied to the soil around the plant stem by a drenching treatment and arranged in a randomised complete block design (RCBD) with five replicates at 5-cm-radius next to the stem of the plants and mixed well with soil. The application occurred once per week for the first four weeks after inoculation. Every other day each plant was watered with 250 mL chlorine-free tap water, and half-strength Hoagland nutrient solution was applied once every other week for the duration of the experiment, while NPK was applied as previously outlined (**Chapter 5; Section 5.2.2.2**).

6.2.3. Data collection

6.2.3.1. Growth parameters

Fifty-six days after nematode inoculation, shoot height was measured from the crown to the flag leaf's top end (**Sithole et al., 2016**). The shoots were detached from the roots and oven-dried at 52 ºC for 72 h and weighed. Roots were removed from pots, rinsed in water to remove soil, blotted dry using filter paper, and weighed to facilitate the calculation of nematode density per plant's total root system.

6.2.3.2. Determination of photosynthetic pigment

The photosynthetic pigments [total chlorophyll (a+b) and carotenoids] were calculated using **Lichtenthaler**'s (**1987**) methodology, as described in **Chapter 5; Section 5.2.1.13**.

6.2.3.3. Protein and superoxide dismutase estimation

Bovine serum albumin (BSA) was used as a standard to calculate total protein (**Bradford, 1976**) as described in **Chapter 5; Section 5.2.1.14**, while superoxide dismutase (SOD) activity was estimated using the method of **Beauchamp and Fridovich** (**1971**) at 560 nm was measured against a blank using a spectrophotometer.

6.2.3.4. Carbohydrate estimation

With slight adjustments, the total carbohydrate content was calculated according to **Sadasivam and Manickam** (**2008**), following the methodology described previously (**Chapter 5; Section 5.2.1.14**).

6.2.3.5. Determination of total phenolic content

Total phenolic content was determined using the Folin-Ciocalteu assay, as described by **Makkar** (**1999**), with modifications using gallic acid as a standard according to the methodology described in **Chapter 3; Section 3.2.3**.

6.2.3.6. Nematode extraction and counting

Nematodes were extracted from roots using the maceration and blending method for 30 sec in 1% NaOCl (**Hussey and Barker, 1973**). The material was filtered through 125, 75, and 25-µm nested sieves, with nematodes collected from the75 and 25-µm mesh sieve. Soil per pot was thoroughly mixed, and a 300 mL soil sample was collected, with nematodes extracted using the sugar-floatation and centrifugation method (**Jenkins, 1964**). Eggs and J2s from root samples and J2s from soil samples were counted from a 10 mL aliquot of each sample using a stereomicroscope. Nematode numbers (J2s) in soil were converted to 2500 mL soil per pot and were used to determine the final nematode population densities (Pf).

6.2.3.7. Gas chromatography-mass spectroscopy (GC-MS) profile of *C. maxima* seeds crude extracts

One gram (1 g) of powdered *C. maxima* seeds were extracted with dichloromethane (DCM), ethyl acetate (EtOAc), and petroleum ether (PE). Extracts were concentrated in a vacuum at 35 ºC using a Buchi Rotary evaporator (Büchi, Flawil, Switzerland) and dried at room temperature under a stream of cold air. About 0.5 mg of each extract was re-dissolved in their respective solvents and analysed for their constituents using Gas Chromatography-Mass Spectrometry (GC-MS).

GC-MS analysis was carried out using a Shimadzu QP-2010 SE Gas Chromatography coupled with an Agilent, 5973 Mass Selective detector driven by Agilent Chemstation software. A Zebron ZB-5MS plus capillary column with 30 m x 0.25 mm internal diameter and 0.25 µm film thickness was used. Ultra-pure helium at a flow rate of 1.0 mL/min was used as a carrier gas with a linear velocity of 37 cm/s. The samples (3 µL) were injected into the column with the injector temperature at 250 ºC. The initial oven temperature was 60 ºC, which was set to increase at a rate of 10 ºC/min to 280 ºC, with a holding time of 3 min at each increment. The mass spectrometer (MS) was operated in the electron ionization mode at 70 eV, and electron multiplier voltage at 1859 V. Other MS operating parameters was: ion source temperature 230 ºC, quadrupole temperature 150 ºC, solvent delay 4 min and scan range 50-700 amu. The compounds were identified by directly comparing the analyte's mass spectrum at a particular retention time to that of the reference standards found in the National Institute of Standards and Technology (NIST) library. The area percentage of each component was calculated by comparing its average peak area to the total areas obtained.

6.2.3.8. Data analysis

All reported data were collected at 56-days after nematode inoculation and were subjected to two-way factorial analysis of variance (ANOVA) using SPSS version 24.00 (IBM Corporation Armonk, New York, NY, United States). Where the data were found not to be normal (1-KS), or the variances were not homogenous, discrete nematode data were transformed through $\log_{10}(x + 1)$ (**Gomez and Gomez, 1984**), but untransformed data were reported. Duncan's multiple-range test ($p<0.05$) was performed to separate the means.

6.3. Results

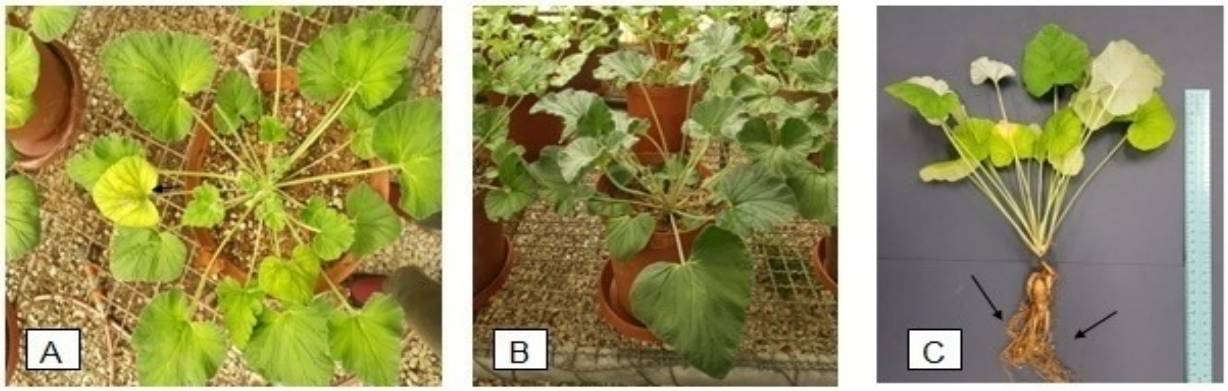

Figure 6.1. The effect of *M. incognita* on susceptible African geranium**.** A = untreated nematode infested plant, B = treated nematode infested plant, and C = the vegetative part and root system with arrows pointing at the developed root galls.

6.3.1. Effect of botanicals on plant growth

Treatments had highly significant effects on shoot height, number of leaves, leaf area, fresh shoot weight, fresh root weight, dry shoot weight and dry root weight, with botanicals contributing 26, 29, 30, 52, 26, 39 and 26%, respectively, in total treatment variation (TTV) of the seven variables (**Appendix 6.1**), whereas the tested application methods significantly affected shoot height, number of leaves, leaf area, fresh shoot weight, fresh root weight and dry shoot weight contributing 53, 38, 54, 24, 8 and 45%, respectively, in TTV of the five variables but had no significant effects on fresh root weight and dry root weight (**Appendix 6.1**), Furthermore, treatments applied as drenching or ground meal had significant effects on the seven plant variables assessed, contributing 18, 32, 13, 22, 57, 15 and 55% respectively, in TTV. Relative to the untreated control, treatments had stimulatory effects on the overall plant growth of *Pelargonium sidoides* (**Figure 6.1; Table 6.1**). There was a significant (p<0.05) increase in the number of leaves, shoot length, leaf area, fresh and dry matter of plants treated with 5, and

2.5 $mgmL^{-1}$ and 10 g/pot treatments over control plants. All growth parameters were significantly increased in nematode-infected plants that were treated with *C. maxima* (T8) when compared with nematode-infected control plants (**Table 6.1**).

Table 6.1. Effects of application of different botanicals on growth of *Pelargonium sidoides* 56-days after nematode application.

Treatments	Plant species	No. of leaves	Leaf area (cm^2)	Shoot length (cm)	Fresh root weight (g)	Fresh shoot weight (g)	Dry root weight (g)	Dry shoot weight (g)
10 g	T1	104 ± 4.01^{a}	$49.14\pm0.78^{e-i}$	$32.43\pm0.57^{b-d}$	$12.39\pm0.49^{h-j}$	89.42 ± 2.52^{a}	2.86 ± 0.18^{c}	12.54 ± 0.29^{a}
	T2	$34\pm0.27^{i-m}$	$57.75\pm0.57^{b-f}$	$24.33\pm1.37^{g-k}$	$13.78\pm0.84^{g-j}$	$34.99\pm2.69^{e-i}$	$3.18\pm0.26^{g-i}$	$4.96\pm0.85^{f-i}$
	T3	$43\pm2.13^{d-h}$	$68.66\pm1.37^{b-d}$	33.33 ± 1.02^{bc}	$16.29\pm4.15^{d-i}$	$39.66\pm1.93^{d-g}$	$3.76\pm0.33^{d-i}$	$6.01\pm0.19^{d-g}$
	T4	60 ± 7.31^{b}	$49.60\pm1.02^{e-i}$	$29.53\pm0.79^{c-f}$	$15.04\pm2.38^{f-j}$	42.09 ± 2.99^{de}	$3.47\pm0.11^{e-i}$	6.56 ± 0.13^{d}
	T5	$42\pm1.25^{e-i}$	$55.32\pm0.79^{c-f}$	21.50 ± 0.29^{jk}	$22.98\pm2.09^{b-d}$	$39.62\pm1.07^{d-h}$	$5.30\pm0.11^{a-d}$	$4.69\pm0.39^{g-j}$
	T6	$33\pm0.94^{j-m}$	$41.94\pm0.29^{f-j}$	$30.17\pm1.03^{c-e}$	$13.66\pm3.27^{g-j}$	$27.38\pm5.08^{i-k}$	$3.15\pm0.47^{g-i}$	$5.35\pm0.18^{d-h}$
	T7	$40\pm0.72^{f-j}$	$71.28\pm1.03^{a-c}$	38.13 ± 0.33^{a}	30.04 ± 3.35^{a}	54.95 ± 1.28^{bc}	6.93 ± 0.54^{a}	8.44 ± 0.56^{c}
	T8	$49\pm4.97^{c-e}$	86.62 ± 0.33^{a}	33.47 ± 0.35^{bc}	$15.20\pm0.99^{e-j}$	54.57 ± 2.23^{bc}	$3.51\pm0.71^{e-i}$	8.97 ± 0.34^{bc}
5.0 $mgmL^{-1}$	T1	$35.00\pm2.83^{h-m}$	$49.67\pm3.93^{e-i}$	$22.43\pm0.42^{i-k}$	$18.53\pm0.97^{c-h}$	$34.99\pm3.24^{e-i}$	$4.28\pm0.22^{c-h}$	$5.73\pm0.23^{d-h}$
	T2	$38.00\pm3.30^{g-l}$	$38.90\pm4.66^{g-j}$	$25.73\pm1.43^{f-i}$	$20.64\pm3.17^{b-g}$	$39.36\pm1.71^{d-h}$	$4.76\pm0.73^{b-g}$	$4.92\pm0.35^{f-i}$
	T3	$36.00\pm0.94^{h-m}$	$48.39\pm1.82^{f-i}$	$22.23\pm0.43^{i-k}$	30.20 ± 3.93^{a}	41.98d±2.03^{e}	6.97 ± 0.50^{a}	$5.37\pm0.91^{d-h}$
	T4	$38.00\pm0.94^{g-l}$	$35.29\pm1.59^{h-k}$	$23.33\pm\pm0.97^{h-k}$	11.03 ± 0.78^{ij}	$30.87\pm0.50^{h-j}$	2.54 ± 0.09^{i}	3.76 ± 0.18^{ij}
	T5	28.67 ± 3.07^{m}	$42.28\pm4.22^{f-t}$	$26.63\pm0.57^{e-h}$	$14.80\pm1.93^{f-j}$	$36.41\pm4.51^{d-h}$	$3.42\pm0.11^{e-i}$	$4.91\pm0.45^{f-i}$
	T6	$42.00\pm1.41^{e-i}$	$48.42\pm1.25^{f-i}$	$21.37\pm1.51^{j-l}$	$22.11\pm0.76^{b-e}$	$38.41\pm1.52^{d-h}$	$5.10\pm0.26^{b-e}$	$4.82\pm0.18^{g-i}$
	T7	$48.67\pm3.54^{c-e}$	$34.88\pm0.85^{i-k}$	$21.87\pm1.48^{i-k}$	$14.35\pm2.35^{f-j}$	$40.82\pm3.78^{d-f}$	$3.54\pm0.54^{e-i}$	$5.08\pm0.73^{e-i}$
	T8	52.00 ± 1.70^{bc}	$47.81\pm4.04^{f-i}$	31.90 ± 0.38^{cd}	$21.38\pm2.57^{b-f}$	42.06 ± 2.3^{de}	$4.93\pm0.59^{b-f}$	$6.21\pm0.59^{d-f}$
2.5 $mgmL^{-1}$	T1	$45.67\pm2.13^{c-g}$	$51.48\pm4.32^{e-h}$	33.27 ± 0.43^{bc}	$25.47\pm0.78^{a-c}$	53.72 ± 2.05^{c}	$5.87\pm0.18^{a-c}$	8.41 ± 0.29^{c}
	T2	$48.00\pm1.41^{c-f}$	$65.13\pm5.22^{b-e}$	36.27 ± 3.04^{ab}	26.97 ± 1.12^{ab}	62.87 ± 4.43^{b}	6.23 ± 0.26^{ab}	9.84 ± 0.85^{b}
	T3	$35.00\pm3.30^{h-m}$	$51.38\pm0.77^{e-h}$	$27.43\pm0.63^{e-g}$	$11.82\pm1.42^{h-j}$	$35.39\pm3.06^{e-i}$	2.73 ± 0.33^{hi}	$6.42\pm0.19^{d-e}$
	T4	$46.67\pm1.66^{c-g}$	$35.55\pm2.06^{h-k}$	$24.73\pm0.93^{g-k}$	$12.23\pm0.47^{h-j}$	42.80 ± 1.39^{de}	2.82 ± 0.11^{hi}	$5.84\pm0.13^{d-g}$
	T5	$51.00\pm3.29^{b-d}$	$42.62\pm0.37^{f-j}$	$24.43\pm2.30^{g-k}$	$21.38\pm0.48^{b-f}$	46.27 ± 4.36^{cd}	$4.93\pm0.11^{b-f}$	6.33 ± 0.39^{de}
	T6	29.67 ± 0.72^{lm}	72.67 ± 3.07^{ab}	$23.97\pm0.79^{g-k}$	$12.45\pm2.03^{h-j}$	$31.30\pm1.05^{g-j}$	2.88 ± 0.47^{hi}	$4.42\pm0.18^{h-j}$
	T7	$39.67\pm1.19^{f-j}$	$47.12\pm8.24^{f-i}$	$23.63\pm0.1.63^{g-k}$	$14.04\pm2.5^{g-j}$	$33.16\pm0.98^{f-i}$	$3.24\pm0.54^{f-i}$	$5.51\pm56^{d-h}$
	T8	$30.00\pm2.16^{j-m}$	$54.91\pm4.02^{d-g}$	$29.07\pm1.06^{d-e}$	$19.90\pm3.08^{c-g}$	$34.061.85^{e-i}$	$4.59\pm0.71^{b-g}$	$5.49\pm0.34^{d-h}$
Control	N	19.00 ± 1.24^{n}	21.90 ± 2.09^{k}	17.47 ± 0.59^{l}	8.84 ± 1.08^{j}	19.15 ± 1.15^{k}	2.78 ± 0.21^{hi}	1.28 ± 0.15^{k}
Control	UNI	$38.67\pm1.91^{g-k}$	29.94 ± 3.57^{jk}	20.63 ± 0.78^{kl}	$16.70\pm1.43^{d-i}$	23.96 ± 1.47^{jk}	$3.85\pm0.33^{d-i}$	3.32 ± 0.27^{ij}

Columns represent mean ±standard error, n = 5. Different letter(s) indicate significant differences ($p<0.05$) between treatments as separated by Duncan's Multiple Range Test. T1 = *P. sidoides*, T2 = *P. africana*, T3 = *S. aculeastrum*, T4 = *V. colorata*, T5 = *S. lancea*, T6 = *M. plumbea*, T7 = *C. sylvaticus*, T8 = *C. maxima*, N = nematode-infested control (Untreated control) and UNI = uninoculated control (nematode free plants).

6.3.2. Effect of botanicals in chlorophyll and carotenoid contents

Chlorophyll and carotenoid contents were enhanced in all treated plants (**Table 6.2**). In the inoculated plants, reduction in Chlorophyll a+b and carotenoid contents were 70 and 51%, respectively, when compared to uninoculated (**Table 6.2**). Furthermore, there was a significant enhancement in plant pigmentation (chlorophyll a, b, and a+b and carotenoid) of *P. sidoides* plants treated with either *C. maxima* crude seed extracts or powdered treatments.

6.3.3. Effect of botanicals on biochemical attributes of African geranium

The protein, superoxide dismutase (SOD) and total phenolics content was increased in nematode-infected control by 1.45, and 1.45-fold and 331%, respectively, compared to the nematode free plants, with a reduction in carbohydrate content by 1.25-fold (**Table 6.3**). Relative to the untreated control, all tested botanicals yielded low phenolic levels. However, a slight increase was produced in plants treated with 5.0 $mgmL^{-1}$ of *C. sylvaticus* (T7). On the other hand, seedlings treated with *C. maxima* (T8) at 5.0 $mgmL^{-1}$ and 10 g significantly reduced the biochemical content, however, stimulated protein, SOD, and carbohydrate contents were observed on plants treated with 2.5 $mgmL^{-1}$ (**Table 6.3**).

Table 6.2. Effect on Chlorophyll a, b, and a + b and carotenoid contents when plants were treated with different powered plant material against root-knot nematodes.

Trt	Chlorophyll a µg g^{-1} FW	Chlorophyll b µg g^{-1} FW	Chlorophyll a+b µg g^{-1} FW	Carotenoid µg g^{-1} FW	Chlorophyll a µg g^{-1} FW	Chlorophyll b µg g^{-1} FW	Chlorophyll a+b µg g^{-1} FW	Carotenoid µg g^{-1} FW	Chlorophyll a µg g^{-1} FW	Chlorophyll b µg g^{-1} FW	Chlorophyll a+b µg g^{-1} FW	Carotenoid µg g^{-1} FW
	GPM (10 g)				2.5 $mgmL^{-1}$				5.0 $mgmL^{-1}$			
N	353±0.6^{f}	108±0.2^{e}	461±0.5^{f}	105±0.3^{d}	353±0.6^{h}	108±0 2^{d}	461±0.5^{f}	105±0.3^{h}	353±0.6^{h}	108±0.2^{f}	461±0.5^{f}	105±0.3^{d}
U	920±22^{d}	598±29^{ab}	1518±50de	215±1.4^{e}	920±22^{d}	598±29^{a}	1518±50^{bc}	215±1.4^{f}	920± 22^{f}	598±29^{bc}	1518±50^{cd}	215±1.4^{c}
T1	1029 ±23^{b}	571±35^{ab}	1601±58^{cd}	314 +2.2^{ab}	944±27^{cd}	613±61^{a}	1557±87^{abc}	245±1.4^{d}	1057±3^{cd}	418±2de	1475±4^{d}	263±0.6^{b}
T2	1178±19^{a}	706±51^{a}	1884±70^{a}	338±11.7^{a}	1104±15^{a}	594±37^{a}	1698±52^{a}	273±0.2^{b}	770±21^{g}	501±48^{cd}	1271±70^{e}	230±0.8^{c}
T3	974±8^{c}	551±22^{b}	1525±29de	293±0.7^{b}	1005±24^{bc}	611±54^{a}	1617±78^{ab}	264±0.3^{c}	1219±7^{a}	777±73^{a}	1996±65^{a}	318±19^{a}
T4	1012±5^{b}	697±84^{a}	1710±83^{bc}	243±21 5^{cd}	725±36^{g}	301±13^{c}	1027±23^{e}	227±3.7^{e}	1126±10^{b}	687±24^{ab}	1814±34^{b}	317±1.5^{a}
T5	1057±11^{b}	673±29^{ab}	1731±39^{abc}	322±1.1^{a}	889±1de	535±2^{a}	1424±2^{c}	233±0.1^{e}	982±23^{e}	625±54^{bc}	1607±76^{cd}	259±2.7^{b}
T6	814±8^{e}	585±18^{ab}	1399±26^{e}	222±0.4^{de}	819±10^{f}	416±19^{b}	1236±28^{d}	244±5.4^{d}	878±1^{f}	345±1^{e}	1223±1^{e}	271±0.1^{b}
T7	877±9^{d}	571±27^{ab}	1448±36de	251±1.4^{c}	849±18^{ef}	571±43^{a}	1421±61^{c}	207±1.7^{g}	1072±10 ^{c}	564±29^{c}	1637±39^{c}	310±0.6^{a}
T8	1165±18^{a}	686±49^{ab}	1852±67^{ab}	336±3 1^{a}	1018±9^{b}	649±22^{a}	1667±30^{ab}	282±0.9^{a}	1015±10de	537±18^{cd}	1552±26^{cd}	267±0.9^{b}

Columns represent mean ±standard error, n = 3. Different letter(s) indicate significant differences (p<0.05) between treatments as separated by Duncan's Multiple Range Test. T1 = *P. sidoides*, T2 = *P. africana*, T3 = *S. aculeastrum*, T4 = *V. colorata*, T5 = *S. lancea*, T6 = *M. plumbea*, T7 = *C. sylvaticus*, T8 = *C. maxima*, N = nematode-infested control (Untreated control) and U = uninoculated control (Nematode free plants).

Table 6.3. Effects of different botanical application on activity of phenols, SOD, protein and carbohydrate contents in *Pelargonium sidoides* roots.

Trt	Protein µg g^{-1} FW	Carbohydrate µg g^{-1} FW	SOD Unit/mg FW	Total phenolic (mg g−1	Protein µg g^{-1} FW	Carbohydrate µg g^{-1} FW	SOD Unit/mg FW	Total phenolic (µg mg−1	Protein µg g^{-1} FW	Carbohydrate µg g^{-1} FW	SOD Unit/mg FW	Total phenolic (µg mg^{-1}
	2.5 mgmL^{-1}				5 mgmL^{-1}				GPM (10 g)			
UNI	8.6±0.03^{f}	20.2±0.15^{cd}	0.011±0.001^{i}	6.05±0.29^{i-k}	8.6±0.03^{f}	20.2±0.15^{d}	0.011±0.001^{i}	6.05±0.29^{i-k}	8.6 ± 0.03^{f}	20.2±0.15^{a}	0.011±0.001^{i}	6.05±0.29^{i-k}
N	12.5±0.03^{abc}	16.2±0.09^{d}	0.016±0.001^{d}	26.07±2.11^{b}	12.5±0.03^{a}	16.2±0.01^{e}	0.016±0.001^{g}	26.07±2.11^{b}	12.5±0.03^{b}	16.2±0.09^{a}	0.016±0.001^{f}	26.07±2.11^{b}
T1	8.1±0.05^{f}	21.1±1.09^{cd}	0.032±0.001^{a}	6.22±0.26^{ij}	9.5±0.05^{e}	35.3±0.46^{ab}	0.017±0.001^{f}	25.75±5.26^{b}	6.9±0.05^{h}	14.0±1.30^{a}	0.024±0.001^{e}	9.64±5.64^{gh}
T2	13.2±0.05^{ab}	21.8±0.10^{cd}	0.020±0.001^{c}	4.37±0.06^{jk}	12.0±0.03^{b}	39.1±4.52^{a}	0.039±0.001^{b}	8.07±0.99^{hi}	10.8±0.05^{f}	16.7±0.09^{a}	0.044±0.001^{a}	5.89±0.54^{i-k}
T3	11.9±0.05^{bcd}	63.2±0.59^{a}	0.011±0.001^{e}	4.08±0.09^{jk}	12.6±0.03^{a}	23.2±0.85^{c}	0.042±0.001^{a}	19.82±3.77^{de}	10.2±0.05^{g}	14.8±0.56^{a}	0.029±0.001^{c}	11.92±2.28^{fg}
T4	10.5±0.03^{de}	29.1±8.40^{bc}	0.011±0.001^{e}	6.12±0.33^{i-k}	9.7±0.03^{e}	12.7±0.63^{e}	0.035±0.001^{c}	8 22±0.89^{hi}	11.0±0.03^{e}	13.3±0.64^{a}	0.036±0.001^{b}	13.20±2.34^{f}
T5	11.2±0.03^{cde}	14.7±0.70^{d}	0.001±0.001^{h}	11.51±0.56^{fg}	11.2±0.03^{d}	38.9±1.79^{a}	0.032±0.001^{d}	12.98±0.86^{f}	12.7±0.1^{a}	15.7±2.00^{a}	0.025±0.001^{e}	13.17±0.24^{f}
T6	9.6±0.03^{ef}	19.6±0.46^{d}	0.003±0.001^{f}	4.87±0.20^{jk}	9.7±0.07^{e}	31.1±0.78^{b}	0.020±0.001^{e}	12.24±3.32^{f}	12.3±0.03^{c}	17.6±4.24^{a}	0.025±0.001^{e}	21.54±2.83^{cd}
T7	10.3±0.03^{de}	34.5±0.78^{b}	0.002±0.001^{g}	3.64±0.21^{l}	11.4±0.03^{d}	15.3±1.02^{e}	0.016±0.001^{g}	29.98±4.12^{a}	11.5±0.01^{d}	13.1±3.64^{a}	0.027±0.001^{d}	17.82±3.89^{e}
T8	13.9±1.70^{a}	16.7±0.09^{d}	0.021±0.001^{b}	11.28±0.27^{fg}	11.8±0.03^{c}	15.2±0.09^{e}	0.012±0.001^{h}	8 31±0.15^{hi}	12.7±0.03^{a}	14.0±6.12^{a}	0.011±0.001^{g}	9.49±0.60^{gh}

Columns represent mean ±standard error, n = 3. Different letter(s) indicate significant differences (p<0.05) between treatments as separated by Duncan's Multiple Range Test. T1 = *P. sidoides*, T2 = *P. africana*, T3 = *S. aculeastrum*, T4 = *V. colorata*, T5 = *S. lancea*, T6 = *M. plumbea*, T7 = *C. sylvaticus*, T8 = *C. maxima*, N = nematode-infested control (Untreated control), UNI = uninoculated control (nematode free plants) and SOD = superoxide dismutase.

6.3.4. Effect of botanicals on nematode infection

Effects of the botanicals and application methods were highly significant on the second-stage juveniles (J2s) in soil, eggs and J2s in roots and reproduction factor (Rf), contributing 17, 2, 1 and 5% in total treatment variation (TTV) of the four variables, respectively (**Appendix 6.2**). The treatments had highly significant effects on number J2s in soil, number of eggs and J2s in roots and reproduction factor (Rf), with botanicals contributing 63, 96, 97 and 88% of TTV, respectively, while the application methods contributed 20, 1, 1 and 6%, respectively, in TTV of the four variables. Hence, **Table 6.4** shows that treatments significantly reduced nematode infection in *P. sidoides* after 56-days of infestation. The application of *C. maxima* seeds as either crude extract (drenching) or ground meal reduced the number of eggs and juveniles by 97 and 90%, and 97 and 96%, respectively, at 5 $mgmL^{-1}$ and 10 g compared to the untreated control. The numbers of second-stage juveniles (J2s) and eggs on the root system of *P. sidoides* were less than that of untreated control; hence, similar trends were noted on the numbers of J2s in soil. Potted soil treated with 10 g of *P. africana*, *S. aculeastrum*, *V. colorata*, *M. plumbea*, *C. sylvaticus* and *C. maxima* had the lowest numbers of juveniles.

Table 6.4. Effect of botanical applications on nematode parameters after 56-days of inoculation with an initial population of 1 000 ± eggs and juveniles.

Trt	5 mgmL^{-1}			2.5 mgmL^{-1}			10 g		
	Eggs	**J2s in roots**	**J2s in soil**	**Eggs**	**J2s in roots**	**J2s in soil**	**Eggs**	**J2s in roots**	**J2s in soil**
N	413±2.96^{a}	967±4.16^{a}	1060±4.62^{b}	413±2.95^{a}	967±544.33^{a}	1060±161.19^{b}	413±73.63^{a}	967±4.33^{a}	1060±161.19^{b}
T1	47±2.35^{c}	133±2.19^{c-f}	40±1.77^{c}	20±1.35^{c}	120±3.10^{c-g}	80±2.33^{e}	47 ±14.40^{c}	193±15.85^{b-d}	353 ±23.72^{c}
T2	53±2.36^{c}	73±1.66^{e-g}	47±1.67^{c}	27±1.67^{c}	20±1.35^{fg}	167±2.13^{cd}	26 ±5.44^{c}	27 ±18.85^{fg}	48 ±14.40^{e}
T3	47±1.45^{c}	27±1.67^{fg}	60±1.77^{c}	33±1.45^{c}	7±1.02^{g}	47±1.67^{e}	7 ±5.44^{c}	153±28.28^{c-e}	60±5,44^{e}
T4	47±2.33^{c}	100±2.13^{d-g}	33±1.45^{e}	47±2.42^{c}	40±1.91^{e-g}	87±2.52^{e}	27±5.34^{c}	27±3.99^{fg}	80 ±19.63^{e}
T5	43±2.52^{b}	227±1.66^{bc}	100±2.81^{d}	27±1.46^{c}	67±1.65^{e-g}	120±1.77^{d}	193±23.73^{b}	300±12.29^{b}	1607 ±24.94^{a}
T6	47±1.94^{c}	93±1.78^{d-g}	40±1.35^{e}	27±2.14^{c}	87±2.35^{d-g}	33±1.66^{e}	33 ±14.40^{c}	13 ±28.80^{g}	53 ±5.44^{e}
T7	7±1.67^{c}	47±1.02^{e-g}	113±1.94^{d}	40±2.28^{c}	107±2.05^{d-g}	80±1.91^{e}	47 ±21.77^{c}	67 ±18.86^{e-g}	60 ±23.73^{e}
T8	13±1.23^{c}	27±1.21^{fg}	126±3.90^{d}	53±2.19^{c}	40±21.35^{e-g}	73±1.05^{e}	40 ±24.94^{c}	48 ±21.77^{e-g}	27 ±10.89^{e}

Columns represent mean ±standard error, n = 5. Different letter(s) indicate significant differences (p<0.05) between treatments as separated by Duncan's Multiple Range Test. T1 = *P. sidoides*, T2 = *P. africana*, T3 = *S. aculeastrum*, T4 = *V. colorata*, T5 = *S. lancea*, T6 = *M. plumbea*, T7 = *C. sylvaticus*, T8 = *C. maxima* and N = nematode-infested control (Untreated control).

6.3.5. Gas chromatography-mass spectroscopy (GC-MS) profile of *C. maxima* seeds crude extracts

The gas chromatography-mass spectroscopy (GC-MS) of the *C. maxima* seeds confirmed the presence of various bioactive compounds with different peak area percentages and retention time (**Table 6.5; Figure 6.2a-c**). The GC-MS chromatograms of the extract further revealed the presence of 141 compounds collectively in crude extracts of EtOAc, DCM, and PE, which manifest the presence of several classes of compounds like alkenes, aromatic hydrocarbon, fatty acids, fatty alcohols, esters, alkenes, aldehydes among others, of which 42, 49 and 50 of the compounds were found in EtOAc, DCM, and PE, respectively, with 13 compounds previously reported with nematicidal attributes (**Table 6.6; Figure 6.3**).

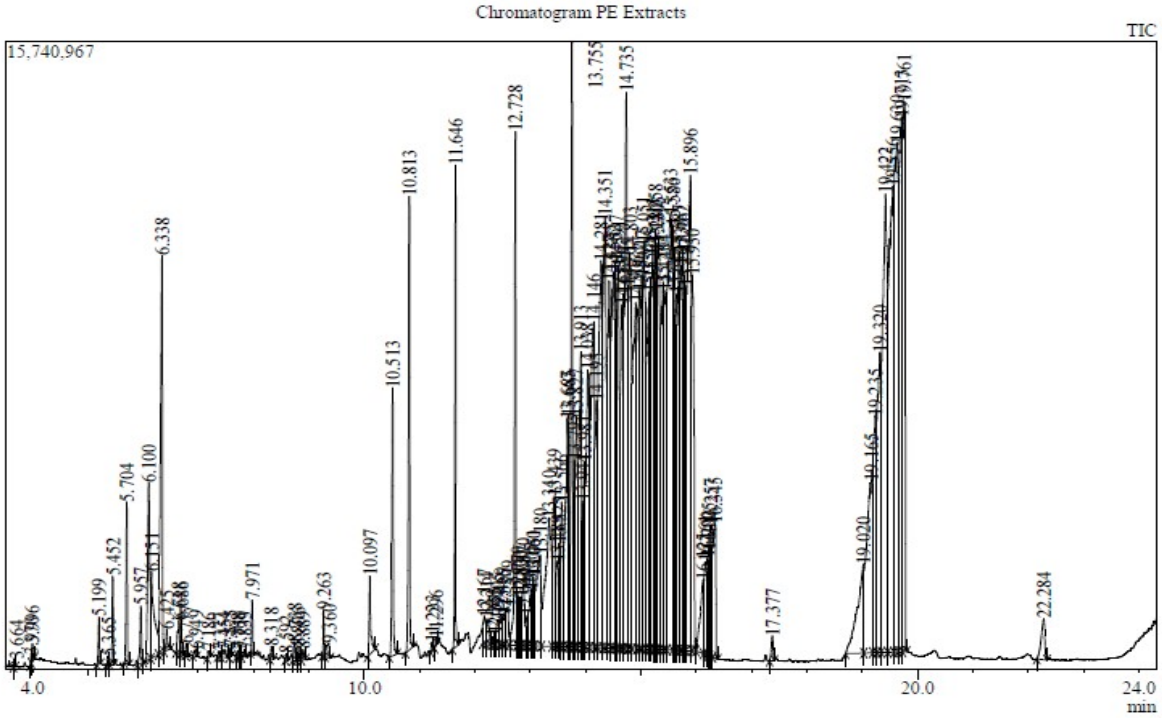

Figure 6.2a. Chromatogram of Petroleum ether (PE) *Cucurbita maxima* seed extracts obtained from GC-MS.

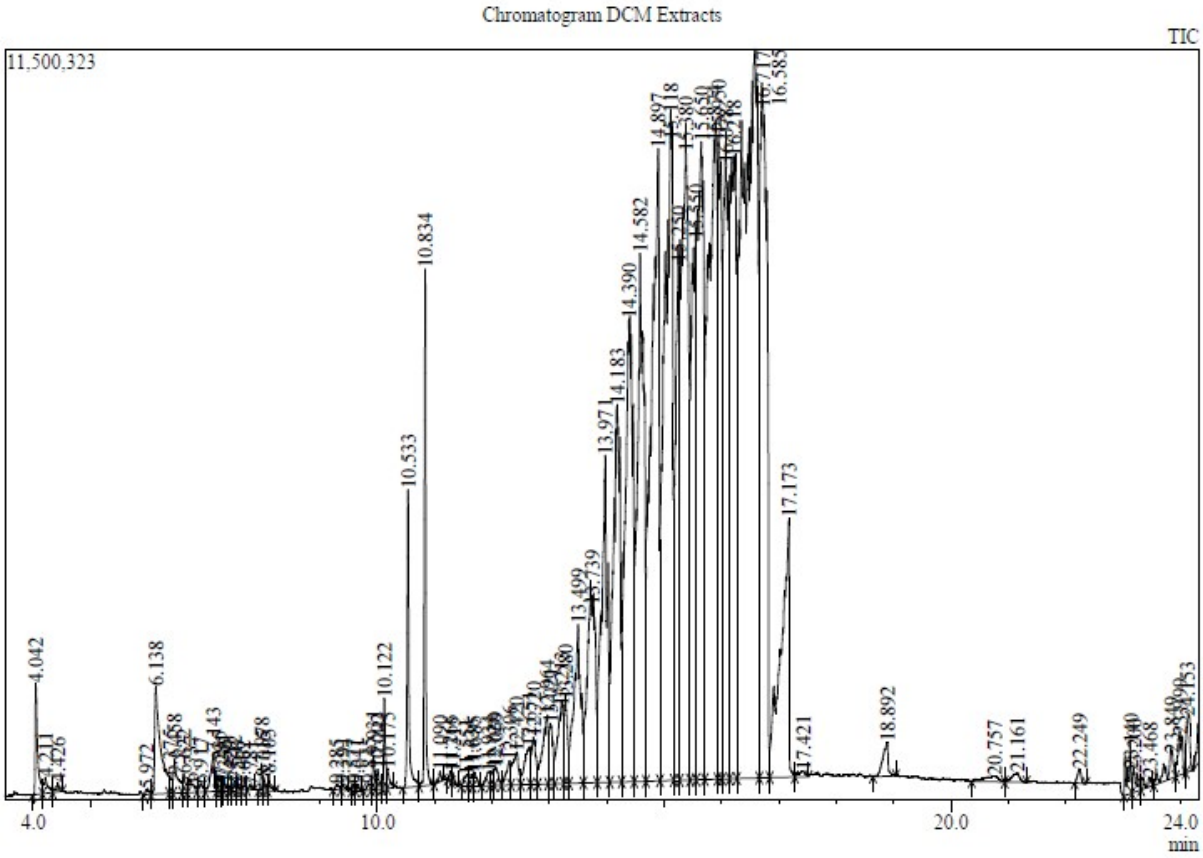

Figure 6.2b. Chromatogram of Dichloromethane (DCM) *Cucurbita maxima* seed extracts obtained from GC-MS.

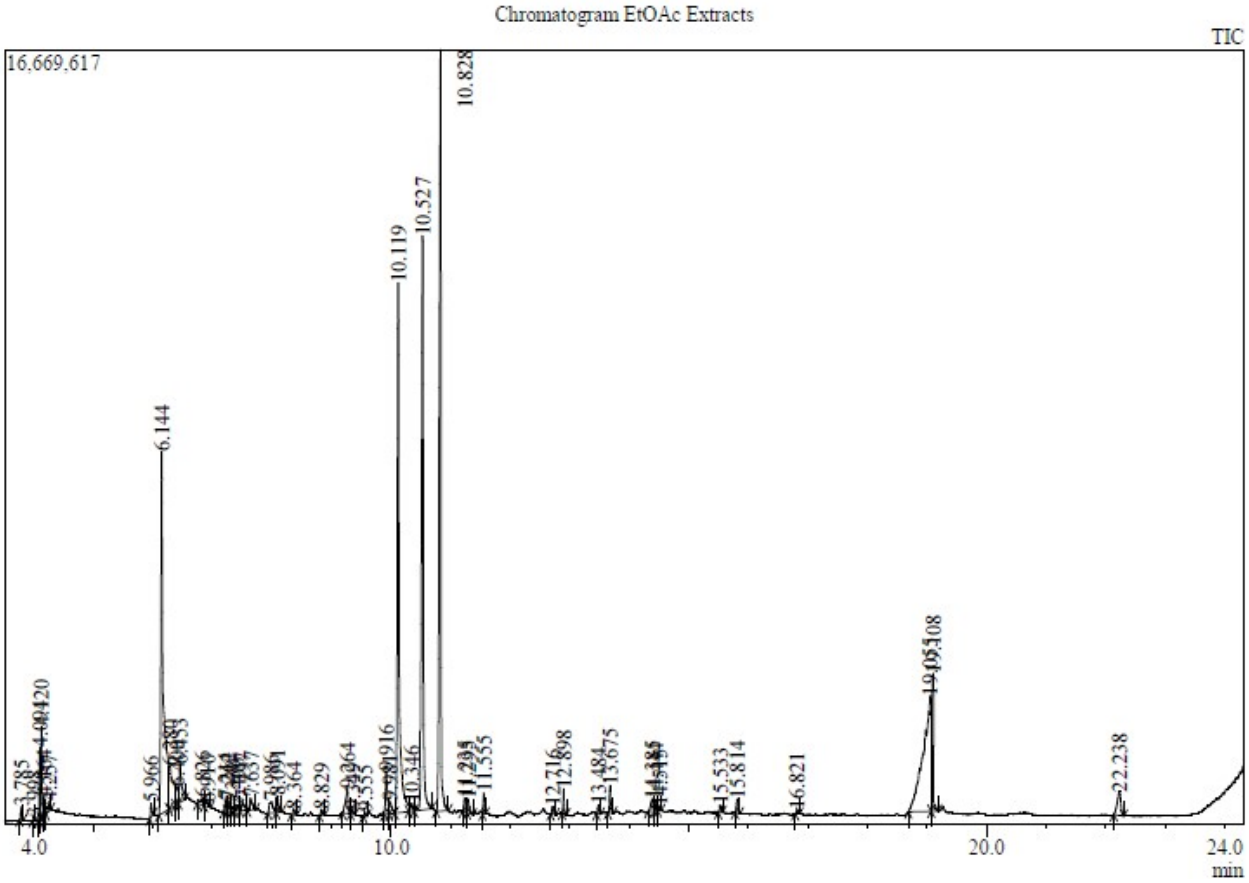

Figure 6.2c. Chromatogram of Ethyl acetate (EtOAc) *Cucurbita maxima* seed extracts obtained from GC-MS.

Table 6.5. Composition of compounds of the dichloromethane extracts obtained from seeds of *Cucurbita maxima*.

	Retention time				Area%			
Peak No.	**PE**	**EtOAc**	**DCM**	**Compound**	**PE**	**EtOAc**	**DCM**	**Organic class**
1	10.513 10.813	10.527	10.533	2,4-Decadienal, (E,E)-	1.59	14.49	0.65	Medium-chain aldehyde
2		3.785		Toluene	-	0.19	-	Aromatic hydrocarbon
3	3.664			Heptane, 3-methyl	0.01	-	-	Alkane
4	3.970	4.094		Heptane,2,4-dimethyl-	0.01	0.94	-	Alkane
5	5.199			Cyclopropane, 2-chloro-1,1,3-trimethyl-	0.09	-	-	Cycloalkane
6	5.365 6.777			Cyclobutanecarboxylic acid, 2-propenyl ester	0.03	-	-	Fatty acid ester
7	5.452			1-Propoxypropan-2-yl 3-methylbutanoate	0.16	-	-	Fatty acid ester
8	5.704			2-Ethoxyethyl 3-methylbutanoate	0.29	-	-	Fatty acid ester
9	5.957			Pentanoic acid, 2-propenyl ester	0.13	-	-	Fatty acid ester
10	6.100			Pentanoic acid, octyl ester	0.60	-	-	Fatty alcohol ester
11	6.151	6.280		2-Heptenal, (E)-	0.49	2.42	-	Medium-chain aldehyde
12	6.338			Cyclopropane, 2-bromo-1,1,3-trimethyl-	1.20	-	-	Cycloalkane
13	6.425	6.453	6.458	1-Octen-3-ol	0.06	1.39	0.17	Fatty alcohol
14	6.648 6.686			Phosphonous dibromide, cyclohexyl-	0.16	-	-	Fatty acid ester
15	6.949	6.826	7.143	Hexanoic acid	0.02	0.31	0.16	Medium-chain fatty acid
16	7.186			3,5-Octadien-2-ol	0.01	-	-	Enone
17	7.377			Malonic acid, 2-ethylbutyl heptyl ester	0.02	-	-	Fatty acid ester
18	7.454			Benzene, 1-ethyl-2,3-dimethyl-	0.05	-	-	M-xylene
19	7.575			Benzene, (1,3,3-trimethylnonyl)-	0.01	-	-	Anisole
20	7.708 7.750			Benzene, 1-ethyl-2,4-dimethyl-	0.02	-	-	Aromatic hydrocarbon
21	7.833			p-Cymene	0.02	-	-	Aromatic monoterpenoids
22	7.971			Undecane	0.09	-	-	Alkane

23	8.318			Benzene, 1,2,4,5-tetramethyl-	0.03	-	-	Aromatic hydrocarbon
24	8.592			Benzene, 1,3-diethyl-5-methyl-	0.01	-	-	Aromatic hydrocarbon
25	8.738			1,3-Cyclopentadiene, 1,2,3,4-tetramethyl-5-methylene-	0.04	-	-	Alkene
26	8.806			Hexadecane, 1-chloro-	0.03	-	-	Aliphatics, halogenated
27	8.889			Benzene, (1,2,2-trimethylpropyl)-	0.03	-	-	Tricyclic aromatic compound
28	9.263			Dodecane	0.08	-	-	Alkane
29	9.360	9.264	9.391	Octanoic acid	0.07	0.93	0.02	Medium-chain fatty acid
30	10.097	10.119	10.122	2-Decenal, (E)-	0.22	15.20	0.18	Medium-chain aldehyde
31	11.223 11.296 12.320 13.755 14.735			2-methyltetracosane	3.85	-	-	Branched alkane
32	11.646			Tetradecane	0.79	-		Alkane
33	12.167 12.214 12.830 13.566 13.795 13.944 13.981 14.038 14.146 14.195 14.351 14.427 14537 14.591 14.679 14.803 14.906 14.967 15.017 15.051 15.211 15.280 15.303 15.425 15.586 15.642 15.687 15.762		11.551 12.420 12.964 13.021 13.739 13.971 14.183 14.390 14.897 15.118 15.380 15.650 15.874 15.950 16.585 17.421 24.153	9,12-Octadecadienoic acid (Z,Z)-	34.66	-	73.54	Lineolic acids and derivatives; long-chain fatty acid
34	12.355			Naphthalene, decahydro-1,5-dimethyl-	0.03	-	-	Aromatic hydrocarbon

35	12.405 12.870 12.940 13.525 13.827 13.913 15.258		11.636 12.651 12.720 13.280 14.582 15.250 16.218	cis-13,16-Docasadienoic acid	4	-	13.67	Long-chain fatty acid
36	12.469			Z,E-3,13-Octadecadien-1-ol	0.11	-	-	Fatty alcohol
37	12.525			Methyl 2-octylcyclopropene-1-heptanoate	0.10	-	-	
38	12.599		16.717	cis-9-Hexadecenal	0.19		5.93	Monounsaturated fatty aldehydes
39	12.728			Heptadecane, 2,6,10,15-tetramethyl-	1.12	-	-	Long-chain alkane
40	12.770			9,17-Octadecadienal, (Z)-	0.08	-	-	Unsaturated aldehyde
41	12.800			Isopropyl linoleate	0.13	-	-	Fatty acid ester and an isopropyl ester
42	13.005 13.051		23.999	Oxacycloheptadec-8-en-2-one, (8Z)	1.33	-	0.06	Alcohol
43	13.060			E,E,Z-1,3,12-Nonadecatriene-5,14-diol	3.10	-	-	Fatty alcohol
44	13.180		23.140 23.200 23.468	Z,Z-8,10-Hexadecadien-1-ol	3.86	-	0.34	Fatty alcohol
45	13.439			cis-7-Tetradecen-1-ol	0.26	-	-	Fatty alcohol
46	13.667			Oleyl alcohol, heptafluorobutyrate	0.97	-	-	Ester
47	13.755			cis-11,14-Eicosadienoic acid, methyl ester	0.58	-	-	Fatty acid methyl ester
48	13.685			(R)-(-)-14-Methyl-8-hexadecyn-1-ol	0.44	-	-	
49	15.150			17-Octadecynoic acid	3.81	-	-	Acetylenic fatty acid
50	15.797			n-Pentadecanol	0.87	-	-	Long-chain fatty alcohol
51	15.930			6-Octadecenoic acid, (Z)-	1.37	-	-	Long-chain fatty acid
52	16.125 17.377			Heptadecanoic acid, heptadecyl ester	0.39	-	-	Fatty acid ester
53	16.161 16.200 16.225 16.257 16.345		17.173	Octadecanoic acid	2.10		2.69	Long-chain fatty acid
54	19.020 19.165 19.235 19.556 19.630 19.761	19.055		l-(+)-Ascorbic acid 2,6-dihexadecanoate	13.32	13.85	-	Ester

55	19.230			Tridecanoic acid	1.79	-	-	Long-chain fatty acid
56	19.422			Eicosane	3.73	-	-	Alkane
57	19.713			Dodecanoic acid	3.13	-	-	Saturated medium-chain fatty acid
58		3.998		1-Octene	-	0.05	-	Alkene
59	3.996	4.120	4.042	Hexanal	0.03	1.35	0.34	Saturated fatty aldehyde
60		4.164		2-Octene, (Z)-	-	0.33	-	Acyclic olefin (alkene)
61		4.257		2-Octene, (E)-	-	0.47	-	Acyclic olefin (alkene)
62		5.966 7.330	5.972	Cyclopentane, 1-ethyl-1-methyl-	-	0.50	0.02	Alkane
63		6.144	6.138	2-Heptenal, (Z)-	-	14.13	0.78	Monounsaturated fatty aldehyde
64		6.400		Cyclopentane, 1-ethyl-2-methyl-	-	0.68	-	Fatty alcohol
65		6.910		1-Nonen-4-ol	-	0.19	-	Fatty alcohol
66		7.214	7.235	3-Octen-2-one	-	0.25	0.02	Enone
67		7.262 7.406		Cyclohexanol, 2,4-dimethyl-	-	0.51	-	Cyclohexanol
68		7.497	7.512	2-Octenal, (E)-	-	0.38	0.03	Medium-chain aldehyde
69		7.637	7.662	2-Octen-1-ol, (E)-	-	0.78	0.02	Fatty alcohol
70		7.986		Cyclobutanecarboxylic acid, 3-pentadecyl ester	-	0.59	-	Ester
71		8.091	8.105	Nonanal	-	0.38	0.04	Saturated fatty aldehyde
72		8.364		1-Octanol, 2-nitro-	-	0.15	-	Fatty alcohol
73		8.829		2-Nonenal, (Z)-	-	0.13	-	Medium-chain aldehyde
74		9.349		5-Undecene, 7-methyl-, (E)-	-	0.09	-	Alkene
75		9.555	9.641	2,4-Nonadienal, (E,E)-	-	0.07	0.01	Medium-chain aldehyde
76		9.916		3-Hexene, 2,2,5,5-tetramethyl-, (Z)-	-	1.18	-	Alkene
77		9.981		Furan, 2,3-dihydro-4-(1-methylpropyl)-, (S)-	-	0.44	-	
78		10.346		Nonanoic acid	-	0.37	-	Medium-chain fatty acid
79		11.295		2-Undecenal	-	0.32	-	Medium-chain aldehyde
80		11.555		9-Octadecene, (E)-	-	0.32	-	Alkene

81		12.716		Z-8-Methyl-9-tetradecenoic acid	-	0.22	-	Fatty acid
82		12.898		Phenol, 2,4-bis(1,1-dimethylethyl)-	-	0.42	-	Phenol
83		13.484		1,7-Nonadiene, 4,8-dimethyl-	-	0.18	-	Alkene
84		13.675		(E)-14-Hexadecenal	-	0.52	-	Medium-chain aldehyde
85		14.385		(E)-13-Docosenoic acid	-	0.49	-	Long-chain fatty acid
86		14.444		9-Octadecyne	-	0.20	-	Alkyne
87		14.515		Z-5-Nonadecene	-	0.07	-	Alkene
88		15.533		Tetradecanoic acid	-	0.18	-	Long-chain fatty acid
89		15.814		1-Heneicosanol	-	0.30	-	Fatty alcohol
90		16.821		1,2-Benzenedicarboxylic acid, bis (2-methylpropyl)	-	0.09	-	Benzoic acid ester
91		19.108	18.892	Pentadecanoic acid	-	3.77	0.23	Long-chain fatty acid
92		22.238		Heneicosane	-	1.31	-	Alkane
93			4.211	1-Octyn-4-ol	-	-	0.08	Alkynyl alcohol
94			6.375	1-Heptene, 5-methyl-	-	-	0.07	Unsaturated aliphatic hydrocarbon
95			6.622	Butyric acid, 2,2-dimethyl-, vinyl ester	-	-	0.07	Acyclic monoterpenoids
96			6.737	Octanal	-	-	0.07	Medium-chain aldehyde
97			6.917	2,3-Octanediol	-	-	0.05	Fatty alcohol
98			7.560	Pentanoic acid, 2-methyl-, anhydride	-	-	0.01	Fatty acid
99			7.781	3,3-Dimethyl-2,4-pentane dione	-	-	0.02	Alpha diketone
100			7.978	Furan, 2,3-dihydro-4-(1-methylpropyl)-,(S)-	-	-	0.05	
101			8.015	Cyclobutanecarboxylic acid, cyclobutyl ester	-	-	0.04	Fatty acid ester
102			9.285	2,4-Dimethylcyclopentanol	-	-	0.03	Alcohol
103			9.641	Oxalic acid, cyclobutyltetradecyl ester	-	-	0.01	Fatty acid ester
104			9.845	Pentylidenecyclohexane	-	-	0.03	Unsaturated hydrocarbon
105			9.931	3-Methyldec-3-ene	-	-	0.08	Alkene
106			10.002	Bicyclo[3.2.0]heptan-2-one, 6-hydroxy-5-	-	-	0.06	

				methyl-				
107			10.175	Oxirane, (3,3-dimethylbutyl)-	-	-	0.06	Ester
108			11.090	Cyclopentanone, 2-(2-octenyl)-	-	-	0.03	Katone
109			11.266	2-Decen-1-ol, (E)-	-	-	0.02	Fatty alcohol
110			11.315	8-Hexadecenal, 14-methyl-, (Z)-	-	-	0.06	medium-chain aldehyde
111			12.020	1,E-11,Z-13-Octadecatriene	-	-	0.05	Alkene
112			12.069	11,14-Eicosadienoic acid, methyl ester	-	-	0.09	Ester
113			12.306	Ethyl 9,12-hexadecadienoate	-	-	0.13	Fatty acid ester
114			15.550	5-Hepten-1-ol, 2-ethenyl-6-methyl-	-	-	3.35	Fatty alcohol
115			16.078	Z-8-Octadecen-1-ol acetate	-	-	4.60	Fatty alcohol ester
116			20.757	3',8,8'-Trimethoxy-3-piperidyl-2,2'-binaphthale	-	-	0.10	
117	22.284		21.161 22.249	2-methyloctacosane	0.25	-	0.15	Alkane
118			10.834	2,4-Decadienal	-	18.99	0.90	Medium-chain aldehyde
119				2-methylhexacosane	3.30			
120	15.175			4H-Cyclopentacyclooctene, decahydro-	0.58			
				Total	100	100	100	

Table 6.6. Compounds found in seeds of *Cucurbita maxima* that are known to cause nematicidal effects against various plant-parasitic nematodes.

Compounds	**Common name**	**Formula**	**References**
Octadec-9-enoic acid z (cis)	Oleic acid	$C_{18}H_{34}O_2$	**Tarraf et al. (2019)**
Octadecanoic acid	Stearic acid	$C_{18}H_{36}O_2$	**Zhang et al. (2012)** **Ntalli et al. (2020b)**
17 octadecynoic acid	Linoleic acid	$C_{18}H_{32}O_2$	**Zhang et al. (2012)** **Ntalli et al. (2020)**
Octanoic acid	Caprylic acid	$C_8H_{16}O_2$	**Zhang et al. (2012)** **Ntalli et al. (2020b)**
Dodecanoic acid	Lauric acid	$C_{12}H_{24}O_2$	**Zhang et al. (2012)** **Ntalli et al. (2020b)**
Tetradecanoic acid	Myristic acid	$C_{14}H_{28}O_2$	**Zhang et al. (2012)** **Ntalli et al. (2020b)**
Pentadecanoic acid	Pentadecylic acid	$C_{15}H_{30}O_2$	**Sharm et al. (2018)**
Hexanoic acid	Caproic acid	$C_6H_{12}O_2$	**Zhang et al. (2012)** **Ntalli et al. (2020b)**
1-octanol	Octanol	$C_8H_{18}O$	**Shivakumara et al. (2018)**
1-octanal	1-caprylaldehyde	$C_8H_{16}O$	**Shivakumara et al. (2018)**
2,4-Decadienal,(E-E)	-	$C_{10}H_{16}O$	**Ntalli et al. (2016)** **Caboni et al. (2012)** **Aissani (2014)**
2-Decenal, (E)	-	$C_{10}H_{18}O$	**Caboni et al. (2012)** **Aissani (2014)**

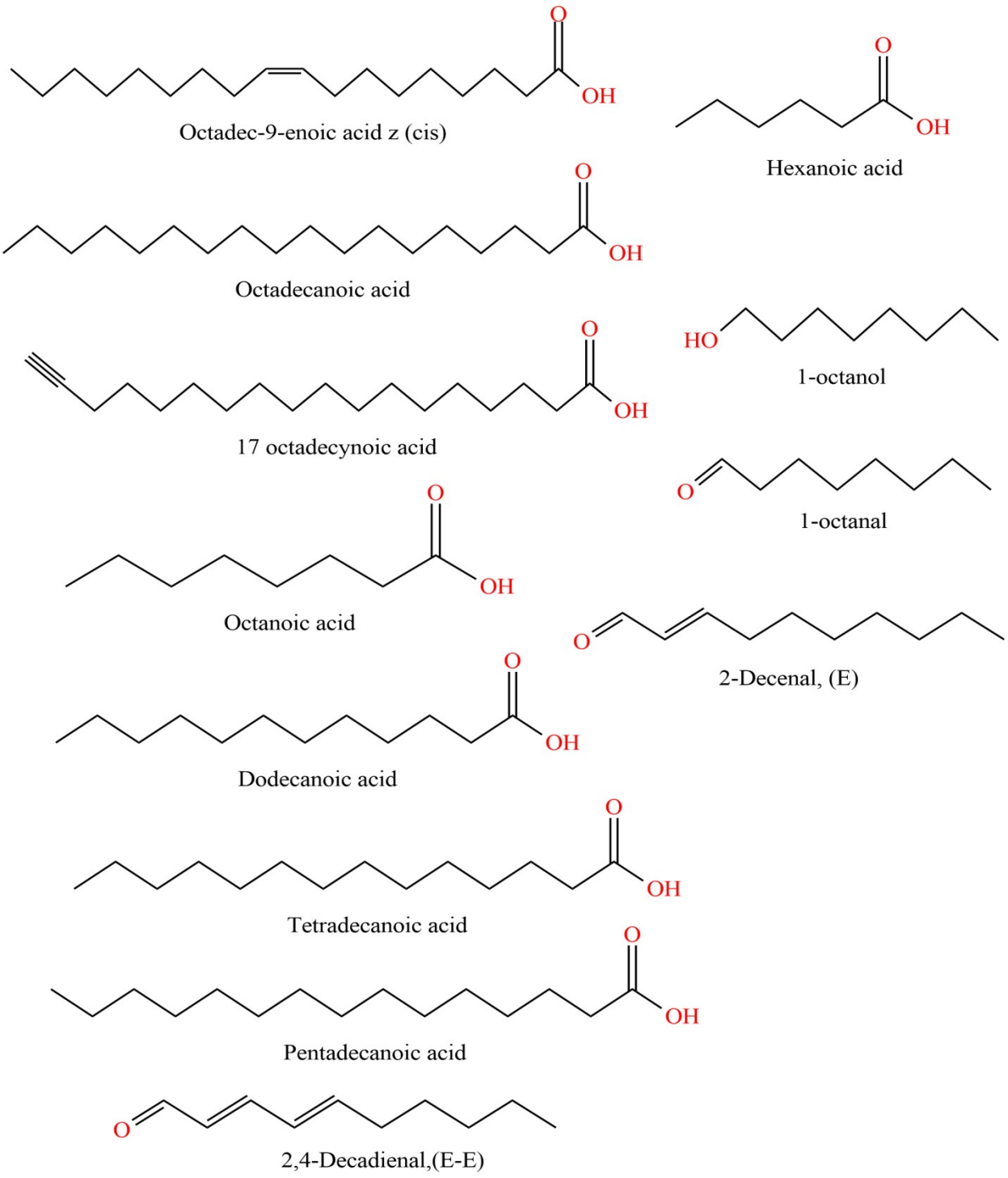

Figure 6.3. Structural compounds found in seeds of *C. maxima* that are known to have nematicidal properties against various plant-parasitic nematodes.

6.4. Discussion

In the present study, treatments increased plant growth variables compared with untreated, inoculated control. The use of botanicals as either in-ground or extract form stimulated plant growth when compared to the nematode-free plants. Hence, several studies have shown that the use of organic plant material significantly controls plant diseases and substantially improves plant growth (**Sithole et al., 2016; Mokrini et al., 2018; El-Deriny et al., 2020**). In addition, the present study also revealed that untreated control plants had a significantly lower total chlorophyll and carotenoid content compared to treated plants. A reduction in chlorophyll from *M. javanica* infected African geranium has been reported by **Sithole et al.** (**2016**), with the opposite being observed after the application of *Cucumis myriocarpus* crude extracts. Similar results were also reported by **Khanna et al.** (**2019b**), where they revealed that nematode-infected seedlings of tomato showed reduced levels of plant chlorophyll, carotenoids, and xanthophylls. Relative to both uninoculated and untreated control, the current study revealed that treatments enhanced the levels of chlorophyll b; this could be due to a tolerance defense mechanism to nematode infection induced by the treatments. **Tanaka et al.** (**1998**) stipulated that chlorophyll a is transformed into chlorophyll b through chlorophyllide a oxygenase (CAO) activity on plants grown in a suitable condition. However, under stressed conditions, CAO activity is damaged, and the synthesis of chlorophyll b declines (**Tanaka et al., 1998**). Hence, this could be the reason for the low chlorophyll content in control plants in the present study.

The current findings further showed that untreated plants had a higher accumulation of protein, carbohydrate, and total phenolic contents compared to those treated with botanicals. There is no disparity between this result and the prior findings of **Vaitheeswaran et al.** (**2011**), who reported

that *M. incognita* infected tissues of *Hibiscus cannabinus* (kenaf) contained higher proteins, reduced sugar levels, and total energy. The increase in phenolic contents observed within the untreated control could be due to the release of conjugated phenols from the glycosidic compounds produced by the plants through actions of hydrolytic enzymes during the nematode colonisation of the plant roots (**Pandey et al., 2016**). Nematode-infected plants have been reported to have increased total phenolic contents, which consequently trigger various pathways that are involved in the synthesis of different chemical compounds, including lignin, that have been proven as a crucial resistance response mechanism (**Mandal et al., 2010; Pandey et al., 2016; Khanna et al., 2019c; Singh et al., 2020**).

The present study further showed that the tested ground material and crude extracts of *C. sylvaticus*, *V. colorata*, *M. plumbea* and *C. maxima* significantly reduced numbers of *M. incognita* juveniles and eggs on *P. sidoides*; similar results have been noted in **Chapter 5** for tomato. The observed nematicidal effects of *V. colorata*, *M. plumbea* and *C. maxima* powdered plant material could be attributed to their richness in anthelmintic compounds as discussed in **Chapter 5**. Furthermore, **Chapter 4** demonstrated that higher concentrations of *C. maxima* and *C. sylvaticus* caused 100% J2s paralysis (immobility) under *in vitro* conditions at 5 mg/mL. **Sithole et al. (2021**) also demonstrated the inhibition of egg hatching with increasing concentrations of *C. maxima*, *P. africana*, *P. sidoides*, *C. sylvaticus*, *S. aculeastrum*, *V. colorata*, *S. lancea* and *M. plumbea* methanolic crude extracts.

In the present study, the crude extracts and ground material of *C. maxima* seeds were highly active against *M. incognita*. The nematicidal component could be due to different secondary

metabolites in the seeds (**Chapter 3; Sithole et al., 2021**). The GC-MS results showed that *C. maxima* seeds contain various compounds from several classes of compounds like alkanes, aromatic hydrocarbon, fatty acids, fatty alcohols, esters, alkenes, and aldehydes. Out of the 42, 49, and 50 compounds identified from the crude extracts of EtOAc, DCM, and PE, respectively 13 compounds have been reported to consist of nematicidal attributes. The promising *in vitro* and *ex-situ* nematicidal activity observed from *C. maxima* seeds in this study can be linked to the well reported aldehydes compounds (2,4-decadienal, (E-E) and 2-decenal, (E)). These compounds were earlier reported by **Caboni et al.** (**2012**), **Aissani** (**2014**) and **Ntalli et al.** (**2016**) as nematicidal agents against *M. incognita*, *M. javanica,* and *M. arenaria* under *in vitro* and greenhouse experiments. Furthermore, *C. maxima* exhibited several fatty acid compounds (Octadec-9-enoic acid z (cis), Octadecanoic acid, 17 octadecynoic acid, octanoic acid, dodecanoic acid, tetradecanoic acid, pentadecanoic acid and hexaonoic acid) that have been previously reported to be promising nematicidal agents. The exposure of *M. incognita* to 2 mmol/L of oxalic acid exhibited 100% juvenile mortality at one day after treatment and subsequently suppressed egg hatching by 95.6% at seven days after treatment (**Jang et al., 2016**). Oxalic acid demonstrated similar nematicidal activity against *M. hapla* (**Jang et al., 2016**). Compounds such as hexadecenoic acid (palmitoleic acid), 4-hydroxy-4-methyl-2-pentanone, hexadecanoic acid (palmitic acid), pentadecanoic acid, diethylhexyl phthalate, octadecene, cyclohexadiene-1-one and 1,2-benzenedicarboxylic acid produced by rhizobacteria *Pseudomonas jessenii* and *Pseudomonas synxantha* showed toxic effects against second-stage juveniles of the root-knot nematode, *M. incognita* (**Sharma et al., 2018**). **Stadler et al.** (**1994**) reported nematicidal activity in cultures of *Basidiomycetes*, *Pleurotus pulmonarius* and *Hericium coralloides* against *Caenorhabditis elegans*. Their work further illustrated that

isolated compounds from cultures of *P. pulmonarius* exhibited nematicidal effects against *C. elegans,* with linoleic acid being the most active compound. At the same time, nematicidal activity was also observed from a mixture of fatty acids, with linoleic, palmitic, and oleic acid as the main compounds, obtained from *H. coralloides* showing both repellent and nematicidal activity towards *C. elegans* (**Stadler et al., 1994**).

Another study by **Mamiya** (**1990**) investigated the nematicidal effect of fatty acids (oleic, linoleic, stearic, and palmitic acid) and reported that oleic acid increased the reproduction rate and survivability of *Bursaphelenchus xylophilus*, while stearic acid enhanced the production rate of *B. xylophilus* but showed no significant effect on the survivability of the nematode population. On the other hand, their results further showed that linoleic acid affected the population of *B. xylophilus* only at a high concentration (10 mg/mL). In contrast, palmitic acid did not affect *B. xylophilus*. **Mamiya** (**1990**) further reported that oleic acid had no effect against the pathogenic nematode *Aphelenchoides besseyi*. **Stadle et al.** (**1994**) showed that fatty acids linoleic, myristic, palmitoleic, and oleic had significantly nematicidal inhibitory properties against *C. elegans*. **Dlian et al.** (**1994**) reported similar findings when using pentanoic acid against *M. incognita*. A study by **Davis et al.** (**1997**) indicated that fatty acid esters (methyl and ethylene glycol esters of pelargonic acid) significantly reduced soybean cyst nematode and root-knot nematode infestation.

An *in vitro* and greenhouse study conducted by **Tarraf et al.** (**2019**) showed the nematicidal effect of *Citrullus colocynthis* (bitter apple) seed oil against *M. incognita* with linoleic acid methyl ester and oleic acid being the main fatty acids compounds, followed by other fatty acids

such as erucic acid methyl ester, palmitic and stearic acid. **Zhang et al.** (**2012**) reported that fatty acids such as butyric, caprylic, capric, lauric, myristic, palmitic, and oleic acid significantly reduced *M. incognita* reproduction rate on *Cucumis sativus* (cucumber). Their work further shows that caproic, caprylic, capric, lauric, myristic, and palmitic acid caused higher significant mortality on second-stage juveniles. Hexanoic acid and acetic acid isolated from *Melia azedarach* (chinaberry tree) fruit caused J2s paralysis and inhibited *M. javanica* egg hatching (**Ntalli et al., 2020b**). Palmitic and linoleic acid isolated from exudates of *Ricinus communis* (castor) roots exhibited repellent properties towards *M. incognita* (**Dong et al., 2018**). A higher concentration of lauric acid isolated from *Chrysanthemum coronarium* (crown daisy) repelled *M. incognita*, on the contrary, at low concentration, lauric acid behaved as an attractive agent (**Dong et al., 2018**). In another study, isoamyl alcohol, 1-butanol, and 2-butanone were attractive to second-stage juveniles (J2s) across a wide range of concentrations, whereas J2s were repelled when exposed to 1-octanol concentrations (**Shivakumara et al., 2018**). Undiluted benzaldehyde repelled to J2s despite being attractive at lower concentrations (**Shivakumara et al., 2018**).

Some of the compounds identified in *C. maxima* have been previously identified in other Cucurbita species (**Veličković et al., 2015; Elhassan et al., 2018**). Pumpkin seeds are mainly consumed for nutritional and medicinal benefits. Furthermore, pumpkin seeds are known to contain anthelmintic agents, which can destroy both the parasite and the eggs (INTERNAL PARASITES (worms) - Source-it Mnandi (weebly.com)). However, unlike the most utilized anthelmintic herbs such as cloves and wormwood, to our knowledge, pumpkin seeds have not been investigated against plant-parasitic nematodes. Therefore, this is the first study to report on the nematicidal activity of *C. maxima* seeds under *in vitro* and greenhouse

conditions. Out of these compounds, several have already been reported for their nematicidal properties (**Table 6.6**). Fatty acids, in particular, have revealed promising nematicidal properties against PPNs. The synergistic effect of these compounds might have caused the high nematicidal activity shown by the extract and ground material of *C. maxima* seeds against *M. incognita*. These results, therefore, offer cogent evidence that could strengthen the reason behind the evaluation of *C. maxima* seeds to mitigate the populations of *M. incognita.*

6.5. Conclusions

Due to their rapid biodegradability and eco-friendly attributes, natural products in pest management are significantly gaining popularity. The current study was designed to contribute to the growing scientific knowledge in the management of nematodes using biological strategies. Local farmers may favor botanical nematicides over synthetic nematicides due to their availability, action specificity, cost-effectiveness, and overall safety. Hence, the findings from this study showed that *Cucurbita maxima* seeds from fruits of cv. Hokkaido, also known as Kuri are rich in promising and potent bioactive compounds that could help control the root-knot nematode (*Meloidogyne incognita)*. Biologically active nematicidal compound(s) derived from plants can be evaluated for their *in vitro* and *ex-situ* nematicidal potentials, which will almost certainly yield improved agricultural outputs.

CHAPTER 7: General conclusions

In general, phytonematodes play a significant role in the kingdom Plantae, particularly balancing the ecosystem (https://www.nrcs.usda.gov/wps/portal/nrcs/detailfull/soils/health/biology). However, plant-parasitic nematodes (PPNs) have been reported to infect various plant species, causing drastic changes in their host plants' physiology and morphology, leading to poor crop quality and yield losses (**Ali et al., 2017; Bernard et al., 2017**). The apparent rapid increases in population growth can cause the establishment of food insecurity for many developing countries. Furthermore, the ongoing withdrawal of synthetic nematicides from the AgroChemical markets poses challenges to the agricultural sector worldwide (**Mashela et al., 2017**). The genus *Meloidogyne* belongs to the family of Heteroderidae. It consists of numerous obligated, biotrophic pathogenic species that feed on the underground parts of their hosts (**Abad et al., 2003**). The root-knot nematode (RKN), *Meloidogyne incognita,* is one of the most damaging RKN and is abundantly found in South African soils. The conventional method of managing RKN incorporates the application of chemical fumigants during pre-and post-planting. However, over the years, these chemical fumigants have been reported to be agents of widespread environmental degradation. Indiscriminate application of chemicals, such as pesticides, has caused incredible damage to the climate and biodiversity. Thus, there has been a paradigm shift towards sustainable methods that are significantly beneficial to the ecosystem (**Mashela et al., 2017**).

Over the years, various researchers have recognized the use of plant material and biocontrols (bacteria and fungi), which has attracted much attention (**Mashela and Nthangeni, 2002**). However, the methods mentioned above are associated with some inherent disadvantages, such

as large amounts of organic material, longer decomposition time, soil pH reduction, and contradictory nematode suppression results (**Seshweni, 2016**). Furthermore, biological controls have a lower specificity; hence, they are regarded as a costly start-up process and cannot completely neutralize the target organism (**Bale et al., 2008**).

Therefore, the current study aimed to explore the potential nematicidal properties of eight plants and two American Type Culture Collection rhizobacteria that have not been tested before, either under *in vitro* or greenhouse conditions. Phytochemical and antioxidant activity was evaluated on eight botanicals and the two rhizobacteria to determine their influence on the physiological and morphological growth of nematode susceptible African geranium and tomato as an integrated pest management strategy. This study also investigated the mutagenic (Ames) effect of the botanicals and their antibacterial (serial-dilution) properties against rhizospheric bacteria strains.

Three different solvents, namely water, methanol, and petroleum ether, were used for the extraction of *Cucurbita maxima* (seeds), *Prunus africana* (leaves), *Pelargonium sidoides* (tuberous roots), *Croton sylvaticus* (leaves), *Solanum aculeastrum* (leaves), *Vernonia colorata* (leaves), *Merwilla plumbea* (bulbs), and *Searsia lancea* (leaves) crude extracts for the antioxidant assay. Out of the eight botanicals, *P. sidoides*, *S. aculeastrum* and *S. lancea* recorded the highest antioxidant activity, while *C. maxima* produced minor activity. Plants that exhibited good antioxidant activity also had higher total phenolic and tannin contents. The botanicals also showed various ranges of phytochemical properties; therefore, these results provided some degree of evidence for their use to treat helminthic nematodes, as previously reported. The

botanicals' mutagenic test demonstrated an increase in the number of His^+ revertants with increasing concentration against TA102 bacterial strain. However, the colony numbers were significantly less than that of the positive control (4NQO). *Vernonia colorata*, *S. lancea* and *C. sylvaticus* crude extracts, on the other hand, substantially inhibited the growth of rhizobacteria (*Bacillus licheniformis*, *Pseudomonas fluorescens,* and isolated unidentified tomato rhizobacteria strains) with minimum inhibition concentration (MIC) values ranging from 0.01 to 0.12 g/mL, confirming their antibacterial properties. The *in vitro* plant growth-promoting properties of the bacterial strains indicated the production of ammonia, indole-3-acetic acid, siderophore and ethylene, and hydrogen cyanide. However, the two rhizobacterial strains had insufficient DPPH radical scavenging activity. *Bacillus licheniformis* produced the highest overall phenolic and flavonoid contents of the two bacterial strains studied. Although their single-treatment greenhouse application did not affect tomato plant growth or phytochemical accumulation, co-treatment improved tomato plant growth parameters.

Cucurbita maxima, Prunus africana, *Pelargonium sidoides*, *Croton sylvaticus, Solanum aculeastrum*, *Vernonia colorata, Merwilla plumbea* and *Searsia lancea* had their nematicidal properties investigated for the first time under *in vitro* and greenhouse conditions. In the *in vitro* study, higher concentrations (0.8 mg/mL) of methanolic plant crude extracts inhibited RKN egg mass hatching, with *C. sylvaticus* and *C. maxima* extract causing apparent immobility in juveniles, but the paralysis was only temporary. The greenhouse study revealed significant inhibition of J2s penetration/establishment on *Solanum lycopersicum* roots, with inhibition of root gall formation in seedlings treated with *V. colorata* and *C. maxima* crude extracts. Furthermore, the greenhouse study indicated that all powdered botanical materials significantly

increased tomato plant growth with a nematodal reproduction factor of less than 1 unit. *Cucurbita maxima*, *P. sidoides*, *C. sylvaticus, S. aculeastrum* and *M. plumbea* combined with *P. fluorescens* or *B. licheniformis* significantly exhibited a synergetic interaction with an increase in plant growth parameters. However, the opposite was observed in African geranium seedlings where co-treatments had antagonistic effects and consequently had increased *M. incognita* populations. At the same time, single treatments enhanced the growth of African geranium and its phytochemical accumulation.

Soil amendments with organic plant material have gained popularity in the management of plant-parasitic nematodes. According to a recent study conducted by **Makhubu et al.** (**2021**), have reported that so far, only 17 plants with nematicidal properties have been reported in South Africa, with *Ricinus communis* (fruits), *Cucumis myriocarpus* (fruits), and *Cucumis africanus* (fruits) being frequently documented. In addition, this study demonstrated the nematicidal properties of eight medicinal plants, with *Merwilla plumbea* (bulbs), *Croton sylvaticus* (leaves), and *Cucurbita maxima* (seeds) being the most active under *in vitro* (**Sithole et al., 2021**) and greenhouse (**Chapter 5 and 6**) conditions. Previous studies have shown that pumpkin seeds have been reported as a herb that contains anthelmintic agents, which can destroy both the parasite and the eggs. The Gas Chromatography-Mass Spectrometry (GC-MS) analysis of the crude extract of *C. maxima* seeds further revealed the presence of 141 compounds in crude extracts of EtOAc, DCM, and PE, with the presence of several classes of compounds like alkanes, aromatic hydrocarbon, fatty acids, fatty alcohols, terpenes, esters, phenols, alkenes, aldehydes, etc., of which 42, 49 and 50 of the compounds were found in EtOAc, DCM, and PE, respectively. Based on these observations, the GC-MS analysis suggested that the

nematicidal effects of *C. maxima* seeds might be due to direct interactions between one or more of these compound classes, which could have impacted the cuticle and metabolism of the nematode.

The GC-MS analysis identified the following compounds; Octadec-9-enoic acid z (cis), Octadecanoic acid, 17 octadecynoic acids, octanoic acid, dodecanoic acid, tetradecanoic acid, pentadecanoic acid, hexaonoic acid, 1-octanol, 1-octanal, 2,4-decadienal, (E-E) and 2-decenal, (E) from the pumpkin seeds, and these compounds have been previously shown to have nematicidal effects. Therefore, the identified compounds can probably result in nematicidal activity. The RKN integrated management protocol was developed through botanicals with and without rhizobacteria in this research. Overall, this research contributed to a partial understanding of pumpkin seeds' efficacy against root-knot nematodes.

The effectiveness of pumpkin seeds in the management of mammal nematodes has been demonstrated. Plant-parasite nematodes remain one of the most prominent pests causing significant losses in most important crops. Recently, the ability of the pumpkin seeds in *Meloidogyne incognita* suppression elicited new evidence that pumpkin seeds contain anti-nematicidal properties against root-knot nematodes. However, the specific compound(s) responsible for nematode inhibition so far still remains unknown. Studies of fractional isolation and testing of the pumpkin seed extracts using preparative-scale high-performance liquid chromatography (preparative-HPLC) have not been conducted, although such results could improve our understanding of the interactions between pumpkin seeds and parasitic nematodes. Therefore, future research should concentrate on isolating the nematicidal compounds using

preparative-HPLC and testing them individually and in binary mixtures to learn more about their synergistic interactions, egg hatch inhibition, and juvenile mortality and mobility activity.

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APPENDICES

Appendix 4.1. Partitioning mean sum of squares for *Meloidogyne incognita* egg hatching inhibition in methanolic plant extracts of the selected botanicals after 24, 48 and 72 h exposure.

Source	24 h			48 h			72 h		
	MS	**TTV (%)**	**P**	**MS**	**TTV (%)**	**P**	**MS**	**TTV (%)**	**P**
Botanicals	870.7	3	0.00*	1559.5	26	0.00	5409.2	15	0.00*
Concentrations	22966.9	95	0.00*	3633.41	61	0.00	28121.8	78	0.00*
Botanicals*Concentrations	226.7	1	0.25^{ns}	538.69	9	0.00	1682.1	5	0.00*
Error	186.1	1		217.63	4		601.6	2	
Total	24250.4	100		5949.23	100		35814.7	100	

Where TTV = Total treatment variation, MS = Mean square, ns = not significant at $p = 0.05$, * = significant at $p \leq 0.05$.

Appendix 4.2. Partitioning mean sum of squares for *Meloidogyne incognita* egg hatching inhibition in aqueous plant extracts of the selected botanicals after 24, 48 and 72 h exposure.

Source	24 h			48 h			72 h		
	MS	TTV (%)	P	MS	TTV (%)	P	MS	TTV (%)	P
Botanicals	1545.76	30	0.00*	1664.39	14	0.00*	2124.1	7	0.01*
Concentrations	2684.55	51	0.00*	9260.3	78	0.00*	26277.2	85	0.00*
Botanicals*Concentrations	538.45	10	0.22^{ns}	586.06	5	0.01*	1627.3	5	0.00*
Error	441.51	9		322.76	3		817.2	3	
Total	5210.27	100		11833.51	100		30845.8	100	

Where TTV = Total treatment variation, MS = Mean square, ^{ns} = not significant at $p = 0.05$, * = significant at $p \leq 0.05$.

Appendix 4.3. Sources of variation as affecting root gall index and number of juveniles on tomato seedlings after innocultion with *Meloidogyne incognita* second-stage juvenile hatch in methanolic extracts.

	Gall index			**Second-stage juvenile**		
Source	**MS**	**P**	**TTV %**	**MS**	**TTV%**	**P**
Replicates	0.09091		1	145.48	2	
Treatments	6.69697	0.00*	93	5600.92	90	0.00*
Error	0.42424		6	481.98	8	
Total	7.21212		100	6228.38	100	

Where TTV = Total treatment variation, MS = Mean square, * = significant at $p \leq 0.05$.

Appendix 4.4. Partitioning mean sum of squares for *Meloidogyne incognita* second-stage juvenile immobility in aqueous plant extracts of the selected botanicals after 24, 48 and 72 h exposure.

	24 h			48 h			72 h		
Source	**MS**	**TTV (%)**	**P**	**MS**	**TTV(%)**	**P**	**MS**	**TTV (%)**	**P**
Botanicals	1899.39	27	0.00*	2281.2	16	0.00*	2854.3	14	0.00*
Concentrations	4609.08	67	0.00*	11660.5	81	0.00*	17191.2	84	0.00*
Botanicals*Concentrations	405.69	6	0.00*	460.1	3	0.00*	473.4	2	0.00*
Error	3.69	0		5.9	0		7.7	0	
Total	6917.85	100		14407.7	100		20526.6	100	

Where TTV = Total treatment variation, MS = Mean square, * = significant at $p \leq 0.05$.

Appendix 4.5. Partitioning mean sum of squares for *Meloidogyne incognita* second-stage juvenile immobility in methanolic plant extracts of the selected botanicals after 24, 48 and 72 h exposure.

	24 h			48 h			72 h		
Source	**MS**	**TTV (%)**	**P**	**MS**	**TTV (%)**	**p**	**MS**	**TTV (%)**	**p**
Botanicals	1899.39	27	0.00*	1263.91	60	0.00*	5207.97	56	0.00*
Concentrations	4609.08	67	0.00*	513.554	24	0.00*	2602.36	28	0.00*
Botanicals*Concentrations	405.69	6	0.00*	314.411	15	0.00*	1475.83	16	0.00*
Error	3.69	0		5.11E-30	2		2.18E-29	2	
Total	6917.85	100		2091.875	100		9286.16	100	

Where TTV = Total treatment variation, MS = Mean square, * = significant at $p \leq 0.05$.

Appendix 5.1. Sources of variation as affecting plant height, stem diameter, fresh shoot weight, dry shoot mass and root length,after inoculation of rhizobacteria (n = 23).

	Plant height			Stem diameter			Root length			Fresh root weight			Fresh shoot weight			Dry shoot weight		
Source	**MS**	**TTV**	**P**	**MS**	**TTV**	**P**	**MS**	**TTV**	**P**	**MS**	**TTV**	**P**	**MS**	**TTV**	**P**	**MS**	**TTV**	**P**
Replicates	9.03	4		0.88431	34		9.583	16		8.8808	16		27.927	62		0.43666	17	
Treatments	168.388	79	0.02*	1.15136	44	0.16^{ns}	30.3732	50	0.26^{ns}	35.2368	63	0.05*	11.9491	27	0.11^{ns}	1.61878	64	0.04*
Error	35.912	17		0.58688	22		20.6444	34		11.9389	21		4.9763	11		0.4689	19	
Total	213.33	100		2.62255			60.6006			56.0565			44.8524			2.52434		

Where TTV = Total treatment variation, MS = Mean square, ^{ns} = not significant at $p = 0.05$, * = significant at $p \leq 0.05$.

Appendix 5.2. Sources of variation as affecting shoot height, stem diameter, fresh shoot weight, dry shoot mass and root length 56-days after application of ground botanicals at three different levels (n = 149).

	Shoot height			Stem diameter			Root length			Fresh shoots weight			Fresh roots weight			Dry shoot weight		
Source	**MS**	**TTV**	**P**	**MS**	**TTV**	**P**	**MS**	**TTV**	**P**	**MS**	**TTV**	**P**	**MS**	**TTV**	**P**	**MS**	**TTV**	**P**
Replicates	16.343	2		0.479	2		16.285	2		5.55	0		20.812	2		0.1661	0	
Botanicals	225.81	26	0.00*	6.2939	29	0.00*	590.019	78	0.00*	1365.31	80	0.00*	674.777	84	0.00*	57.2544	79	0.00*
Concentration	574.406	67	0.00*	13.8419	64	0.00*	109.864	15	0.00*	291.15	17	0.00*	94.499	12	0.00*	12.3732	17	0.00*
Bot*Con	47.229	5	0.00*	0.8934	4	0.00*	26.418	4	0.00*	35.14	2	0.00*	10.628	1	0.04*	2.4599	3	0.00*
Error	10.788	1		0.1613	1		10.489	1		9.6	1		5.987	1		0.2962	0	
Total	874.576			21.6695			753.075			1706.75			806.703			72.5498		

Where TTV = Total treatment variation, * = significant at $p \leq 0.05$.

Appendix 5.3. Sources of variation as affecting number of juveniles and eggs on tomato seedlings 56-days after innocultion with *Meloidogyne incognita and* application of finely ground botanicals at three different levels (n = 149).

	Number of juviniles			Number of eggs		
Source	**MS**	**TTV**	**P**	**MS**	**TTV**	**P**
Replications	57995.6	0		1.39E+07	1	
Botanicals	8,19E+07	100	0.00*	1.09E+09	89	0.00*
Concentrations	196302	0	0.00*	9.85E+07	9	0.00*
Botanicals*Concentrations	88478.9	0	0.00*	1.47E+07	1	0.00*
Error	29334	0		4891925	0	
Total	82302111			1.217E+09		

Where TTV = Total treatment variation, MS = Mean square, * = significant at $p \leq 0.05$.

Appendix 5.4. Sources of variation as affecting shoot height, stem diameter, fresh shoot weight, dry shoot mass and root length of tomato seedlings 56-days after innocution with *Meloidogyne incognita* and treated with ground botanicals of the eight medicinal plants combined with rhizobactria (*Bl* or *Pf)* (n = 109).

Source	Shoot height			Stem diameter			Root length			Fresh shoots weight			Fresh roots weight			Dry shoot weight		
	MS	TTV	P	MS	TTV	P	MS	TTV	P	MS	TTV	P	MS	TTV	P	MS	TTV	P
Replications	42.12	2		0.86536	7		36.75	1		65.32	3		3.973	0		0.959	1	
Bacteria	546.17	23	0.00*	5.86971	46	0.00*	2268.09	77	0.00*	181.1	8	0.05*	572.417	42	0.00*	114.811	68	0.00*
Botanicals	1132.29	48	0.00*	3.41061	27	0.00*	426.12	15	0.00*	1566.22	72	0.00*	649.128	48	0.00*	45.982	27	0.00*
Bacteria*Botanicals	583.97	25	0.00*	1.87412	15	0.01*	179.24	6	0.00*	314.38	14	0.00*	131.181	10	0.00*	4.763	3	0.01*
Error	52.37	2		0.67763	5		22.64	1		44.92	2		6.782	0		1.708	1	
Total	2356.92			12.69743			2932.84			2171.94			1363.481			168.223		

Where TTV = Total treatment variation, MS = Mean square, * = significant at $p \leq 0.05$.

Appendix 5.5. Sources of variation as affecting number of juveniles in soil and roots, number of eggs and the reproduction factor of tomato seedlings after 56-days of inoculation with *Meloidogyne incognita* and treated with powdered plant material of the eight botanicals combined with *Bl* or *Pf* (n = 109).

Source	Number of J2s in soil			Number of eggs in roots			Number of J2s in roots			Rf		
	MS	TTV	P	MS	TTV	P	MS	TTV	P	MS	TTV	P
Reps	3800000	0		701042	1		6163542	2		0.01	0	
Bacteria	3.797E+09	95	0.00*	8.090E+07	57	0.00*	3.063E+08	81	0.00*	2085.77	98	0.00*
Botanicals	9.364E+07	2	0.00*	3.404E+07	24	0.00*	4.238E+07	11	0.00*	21.48	1	0.00*
Bacteria*Botanicals	1.124E+08	3	0.00*	2.467E+07	17	0.00*	1.840E+07	5	0.00*	20.32	1	0.00*
Error	6409649	0		1957621	1		3178893	1		0.02	0	
Total	4.013E+09	100		142268663	100		376422435	100		2127.6	100	

Where TTV = Total treatment variation, MS = Mean square, * = significant at $p \leq 0.05$.

Appendix 6.1. Sources of variation as affecting shoot height, number of leaves, fresh root weight, dry root weight, fresh shoot weight, dry shoot weight and leaf area of *Pelargonium sidoides* seedlings 56-days after inocution with *Meloidogyne incognita*and application of the selected eight botanicals as powdered plant material and drenching treatment (n = 161).

Source	Shoot height			Number of leaves			Leaf area			Fresh shoots weight			Fresh roots weight			Dry shoot weight			Dry root weight		
	MS	TTV	P	MS	TTV	P	MS	TTV	P	MS	TTV	P	MS	TTV	P	MS	TTV	P	MS	TTV	P
Reps	14.811	2		37.9	1		87.48	2		56	1		23.289	5		1.0724	1		1.3634	6	
TAM	377.34	53	0.00*	1503.19	38	0.00*	3036.29	54	0.00*	853.62	24	0.00*	31.988	8	0.15^{ns}	48.7215	45	0.00*	1.9788	9	$0,11^{ns}$
Bot	186.148	26	0.00*	1128.06	29	0.00*	1694.44	30	0.00*	1875 13	52	0.00*	106.675	26	0.00*	42.5175	39	0.00*	5.8049	26	0.00*
TAM*B	126.848	18	0.00*	1252.41	32	0.00*	714.83	13	0.00*	779.27	22	0.00*	238 3	57	0.00*	16.3218	15	0.00*	12.4498	55	0.00*
Error	4.385	1		25.92	1		88.07	2		24.41	1		16.317	4		0.5904	0		0.8961	4	
Total	709.532			3947.48			5621.11			3588.43			416.569			109.2236			22.493		

Where TTV = Total treatment variation, MS = mean square, TAM = Treatment application methods, Bot = Botanicals, ns = not significant at p = 0.05, * = significant at $p \leq 0.05$.

Appendix 6.2. Sources of variation as affecting number of juvenile in soil and root, number of eggs and reproduction factor on *Pelargonium sidoides* seedlings 56-days after innocution with *Meloidogyne incognita*and application of the selected eight botanicals as powdered plant material and drenching treatment (n = 161).

Source	Number of J2s in soil			Number of eggs in roots			Number of J2s in roots			Rf		
	MS	TTV	P	MS	TTV	P	MS	TTV	P	MS	TTV	P
Replications	6306	0		10300	1		20421	1		0.01619	1	
TAM	612573	20	0.00*	5343	1	0.03*	20356	1	0.01*	0.21092	6	0.00*
Bontanicals (B)	1947609	63	0.00*	734210	96	0.00*	1882556	97	0.00*	3.12967	88	0.00*
TAM*B	512306	17	0.00*	12521	2	0.00*	14678	1	0.00*	0.18748	5	0.00*
Error	11884	0		1463	0		4150	0		0.00777	0	
Total	3090678			763837			1942161			3.55203		

Where TTV = Total treatment variation, MS = mean square, TAM = Treatment application methods, Bot = Botanicals, ns = not significant at $p = 0.05$, * = significant at $p \leq 0.05$.