

**CYTOTOXICITY AND GENOTOXICITY OF CUCURBITACIN-CONTAINING
PHYTONEMATICIDES AND RELATED BIOAVAILABILITY IN TOMATO PLANTS**

by

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THESIS

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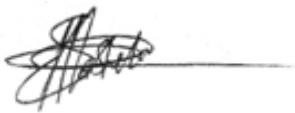
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2025

DECLARATION

I declare that the thesis hereby submitted to the University of Limpopo, for the degree of Doctor of Philosophy in Agriculture (Plant Production) has not previously been submitted by me for a degree at this or any other university; that is my work in design and in execution, and that all material contained herein has been duly acknowledged.



Mashela, T.S., (Mr)

18/09/2025

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DEDICATION

I dedicate this to my loving parents, Prof P.W. Mashela and Mrs R.M. Mashela, and my supportive siblings, Tebelelo, William Jnr and Khensani.

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ABSTRACT

The withdrawal of synthetic chemical nematicides from the agrochemical market has left farmers with limited options to manage plant parasitic nematodes. Currently, the indigenous plants *Curcumis myriocopus* and *Cucurmis africanus* fruits are utilized to develop Nemarioc-AL and Nemafric-BL phytonematicides at the University of Limpopo, South Africa. These noble products are effective on nematode suppression on different crops. The products are environmentally friendly and cost effective compared to synthetic chemical nematicides which cost a fortune. The two products are viewed as a future bionematicide. However, more information is required on safety to non-target organisms which can be negatively affected by the product during application. Although bionematicides are viewed as a potential product, to manage nematodes, less than 1% are currently registered in the agrochemical market. Registration authorities have not formulated the legislative framework for botanicals as alternative agricultural remedies to the withdrawn synthetic chemicals. This study consists of five objectives (i) investigate the bioavailability of Nemarioc-AL and Nemafric-BL phytonematicides in plant tissues and chemical residues in different soil types. (ii) examine whether exposure of earthworms to Nemafric-BL or Nemarioc-AL phytonematicides would affect earthworm protein content. (iii) determine whether exposure of Raw 264.7 macrophage cells to Nemarioc-AL and Nemafric-BL phytonematicides would have cytotoxic effects. (iv) investigate whether short-term dermal and oral exposure to Nemarioc-AL and Nemafric-BL phytonematicides would induce mortality or any structural deformities on earthworms. To achieve these objectives, reliability of measured variables was ensured by using statistical levels of significance ($P \leq 0.05$) and coefficient of determination (R^2), with validity ensured by conducting three independent experiments over time. The bioavailability of cucurbitan A and B was observed in tomato fruits, leaves, stems, roots and soil treated by Nemarioc-AL and Nemafric-BL phytonematicides. At 150 days after application of Nemarioc-AL phytonematicide, the accumulation of cucurbitacin A in tomato fruits was not detected, while cucurbitacin B was detected. Sandy loam (+), had the highest concentration of cucurbitacin B

(0.034 mg/kg), followed by red soil with 0.027 mg/kg, then sandy soil with 0.022 mg/kg and red soil with 0.20 mg/kg. No bioavailability of both cucurbitacin's was detected in the untreated controls. In contrast, the highest cucurbitacin B accumulation was detected in red soil, followed by dark soil, then followed sandy soil and sandy loam (+) with an average mean of 0.034, 0.028, 0.025 and 0.026, respectively. Using the biological indices generated from Curve-fitting Allelochemical Response Dose (CARD) computer- algorithm model a MCSP of Nemarioc-AL phytonematicide on tomato plants inoculated by earthworms was successfully developed at 1.09%, with the overall sensitivity (Σk) value of 1 unit. Nemafric-BL phytonematicide had a computed MCSP of 1.35%, with the overall sensitivity (Σk) value of 2 unit. Proteomics, using one dimensional electrophoresis gel, lower concentrations of Nemarioc-AL phytonematicide 2 and 4% has shown shallow bands, while high concentrations (4, 8, 16, 32 and 64%) showed intensified bands of proteins. The MTT assay was used to assess toxicity of Nemarioc-AL and Nemafric-BL phytonematicides on murine Raw 264.7 macrophages. The increasing concentration of Nemarioc-AL phytonematicide exhibited 50% cytotoxic effects to cell viability of Raw 264,7 cell line at 5 and 10 mg/mL relative to untreated and curcumin treated control. Similarly, Nemafric-BL phytonematicides, showed 50% cytotoxicity at 5 and 10 mg/mL. As the concentrations of Nemarioc-AL and Nemafric-BL phytonematicides decreased from 1.25 to 0.078 mg/mL, the cell viability of Raw 264.7 cell lines were stimulated relative to positive control. Lower concentrations of phytonematicides showed moderate to no cytotoxicity on Raw 264.7 cell lines. While higher concentrations have shown higher degree of cytotoxicity. Lower concentrations (2-8%) of both phytonematicides did not impact the earthworm's viability, while at 16 to 64%, they were no viability observed. Less structural deformities were observed at 2 to 8% concentration, while at higher concentrations have shown more structural deformities indicating sings of toxicity. The major findings for this study were Nemarioc-AL and Nemafric-BL phytonematicides have shown less toxicity at lower concentrations levels, suggesting that it wont affect non-target species. These findings will help the University of Limpopo to further register these products in the agrochemical market.

CHAPTER 1

1.GENERAL INTRODUCTION

1.1 Background

1.1.1. Description of the research problem

Globally, the use of synthetic chemical pesticides in tomato (*Solanum lycopersicum* L.) plants to optimize yield and quality of fruits have raised controversies, including high production costs, non-target organisms, human or animal health and environmental concerns (Ali *et al.*, 2021). The phase-out of synthetic chemical pesticides from the agrochemical markets has left farmers with no options to manage economic pests like root-knot nematodes (*Meloidogyne spp.*) (Sithole, 2021). In response to this crisis, two noble products Nemarioc-AL and Nemafric-BL phytonematicides from the fermented fruit of wild watermelon (*Cucumis africanus* L.) and wild cucumber (*Cucumis myriocarpus* Naud.) were developed as alternatives to manage plant-parasitic nematodes in context of climate-smart-Agriculture (Mashela *et al.*, 2017). The efficacy of the cucurbitacin phytonematicides in suppressing plant nematodes was comparable to that of synthetic chemical nematicides such as velum®, aldicarb® and fenamiphos® (Mashela *et al.*, 2008; Pofu and Mashela, 2022; Seshweni, 2016). Several plant extracts from wild plants used for plant protection purposes, just like those used in traditional medicines, were selected and tested on the basis that they were safe based on the duration they had previously been used by man (Morobe *et al.*, 2012; Ndhlala *et al.*, 2013).

Fruits from the two *Cucumis* species have been utilized in the treatment of liver damage, immune system weakening, lumps, gonorrhea, inflammation, and intestinal

roundworm infections (Mphahlele *et al.*, 2012). Nonetheless, subsequent research has indicated that plant extracts possess the ability to trigger malignant development and/or cytotoxicity in healthy cells of diverse organisms. In certain cases, this has led to human fatalities as well as deaths of non-target species such as earthworms (*Eisenia fetida*) (Falya *et al.*, 2020; Joubert and Sebata, 1982; Ntlhamu *et al.*, 2021).

1.1.2 Impact of the research problem

The use of synthetic chemical pesticides for plant protection purposes to control nematodes, insects, fungi, bacteria, weeds and to preserve quality and yield of produce has been in practice since dawn of commercial agriculture (Anani *et al.*, 2020; Ayilara *et al.*, 2023). Nevertheless, there are drawbacks to synthetic chemical pesticides due to their negative effects on animals, aquatic life, humans and soil biodiversity (Farooq *et al.*, 2019); Ayilara *et al.*, 2023). Despite strict regulations on the use of synthetic nematicides in tomato husbandry, approximately 2 million tons of synthetic chemical pesticides are used worldwide for plant protection purposes to preserve yield or quality of produce, with 45% utilized in Europe, 25% in the US, and 25% elsewhere per annum (Alavanja, 2009; Ali *et al.*, 2021).

The use of indigenous plant extracts to manage pests, is viewed as a future solution to alleviate harmful substances from synthetic chemical pesticides. However, the use of plant extracts requires knowledge on Taxonomic classification of plants, standardised doses to be administered for safety purposes while preserving the used species to avoid extinction. Van Wyk and Prinsloo (2020), reported poisoning cases from plant-based products due to ingestion of toxic traditional medicines resulting in

mortalities. Therefore, despite the long history of use, the potential of plants to cause toxicity should not be ignored.

The plant-based products, Nemarioc-AL and Nemafric-BL phytonematicides, as was demonstrated using the Curve-fitting Allelochemical Response Dose (CARD) computer- algorithm model (Liu *et al.*, 2003; Malebe, 2019; Mashela *et al.*, 2017), have density-dependent growth (DDG) patterns, which are characterised by stimulation (cancerous) and inhibition (cytotoxicity) properties on healthy cells. A wide range of studies had been conducted to study the inhibition properties of cucurbitacins on cancerous cells (Sikander *et al.*, 2016), however, with no success in developing anti-cancer products. Generally, products from plant extracts used for plant protection purposes are designed to inhibit growth of specific pests that pose a threat to agricultural commodities. They exert their effect by targeting metabolic systems which can be similar to humans and soil microorganisms (AL-Ahmadi, 2019). Such toxic chemical compounds, when exposed to animals, can lead to life-threatening complications such as cytotoxicity, mutagenic and carcinogenic active cases (Falya *et al.*, 2020, Morobe *et al.*, 2012; Ndhlala *et al.*, 2013). Accordingly, during pre-clinical registration of lead chemical compounds such as cucurbitacins, the registration authorities would want assurances that the chemicals would, when precautionary measures are taken, not have cancerous, mutagenic or cytotoxic effects on non-target organisms.

1.1.3 Possible causes of research problem

Cucurbitacins are allelochemicals that are produced by plants as secondary metabolites for defense mechanism. Furthermore, as demonstrated using the CARD model, these compounds have a potential to induce cancerous (stimulation) cytotoxicity (inhibition) or mutagenic effects on healthy cells (Liu *et al.*, 2003; Ndhkala *et al.*, 2010; Mashela *et al.*, 2017). In agricultural products and plant protection, the stimulation effects on plant growth are considered a non-issue, whereas inhibition of growth is the main issue (Bango *et al.*, 2019; Malebe, 2019; Sithole *et al.*, 2016). However, for nontarget organisms, the stimulation can be detrimental to health and may result in fatalities. Therefore, it is important to identify cytotoxic and mutagenic substances that can lead to cancer (Mortelmans and Zeiger, 2000; Yapıcı *et al.*, 2021). In products intended for sustainable development, it is imperative that such products be assessed for their potential risks when non-target organisms are exposed in order to have a certain degree of human health assurance (Ndhkala *et al.*, 2010; Ferreira *et al.*, 2022).

1.1.4 Possible solution for research problem

Generally, the efficacy of cucurbitacin phytonematicides was shown to be organism-specific, with smaller animals being highly sensitive to the products than their larger counter parts (Mashela *et al.*, 2020a). In the same token, earthworms were much larger than nematodes, and might require much higher concentrations to suffer damage from the test products. However, these beneficial organisms were highly affected by anthropogenic sources which were toxic to them (Alengebawy *et al.*, 2021). In order to sustain the environment, substances used in agricultural or therapeutic medicines need to be investigated, for any potential risk of toxicity caused by their

active ingredients to non-target organisms and human health according to the South African Act 36 of 1947. Also known as the Fertilizers, Farm Feeds, Agricultural Remedies and Stock Remedies Act (FFFARSRA), regulates the sale, importation, and use of various agricultural products. Therefore, in this study, an investigation of chemical residues of cucurbitacin-containing phytonematicides was carried out in tomato plants exposed to optimized concentrations of Nemarioc-AL and Nemafric-BL phytonematicides. In the same study, the effects on the morphology, proteomics and mortality of the same products on earthworms was investigated. Furthermore, the toxicity of the products was tested using vero and macrosome cells to assess potential toxicity.

1.2 PROBLEM STATEMENT

Fermented fruit extracts were utilized in the manufacture of Nemarioc-AL and Nemafric-BL phytonematicides, respectively (Mashela *et al.*, 2017). These products act as antagonists to well-known plant-parasitic nematodes (Tseke and Mashela, 2018). The evidence for the above-mentioned products' effectiveness in reducing nematode numbers, stimulating nutrient elements and managing toxicity in tomatoes was documented (Bango *et al.*, 2019; Dube, 2016; Shadung, 2016). However, these products are not registered for nematodes management in the agrochemical market. Improper testing of botanical products has limited chances for many biopesticide registrations in the agrochemical market. Similarly, there is no data to explain the bioavailability of these products in crops and if there are any residues translocated into the plant from the exposed soil and what is the effects in terms of toxicity to humans on consumption is any.

1.3 RATIONALE

Tomato plants are planted worldwide because of their fruits, economic value and health benefits (Malherbe and Marais, 2015; Renna *et al.*, 2018). However, most cultivated tomato plants are susceptible to parasitic nematodes, drastically reducing the produce quality and yield (Onkedi *et al.*, 2014). The two noble products, Nemarioc-AL and Nemafric-BL phytonematicides have been shown to suppress nematode numbers on various crops (Mashela *et al.*, 2017, Tseke and Mashela, 2018). Moreover, the cucurbitacin-containing phytonematicides were low-cost products developed from South African native plants that can be easily accessible to subsistence, small scale and commercial farmers in tomato husbandry. However, assessing how cucurbitacin-containing phytonematicides affect organisms that were not the intended targets was important.

Earthworms were a good choice to test the potent toxic substance induced by synthetic chemical pesticides in soil environments. As confirmed by Pelosi *et al.*, (2014), earthworms were non-target creatures that were used as a benchmark for anthropogenic examination of soil microorganisms to determine a substance's potential toxicity. Additionally, according to Datta *et al.*, (2016) and Jansirani *et al.*, (2012), they were the most significant soil organisms in the formation and maintenance of nutrient value by converting organic matter into nutrient-rich vermicomposting. This would provide tomato plants with a significant amount of nutrients in the soil. Therefore, because nematodes and earthworms coexist in the same habitat, it was possible to determine how cucurbitacin-containing phytonematicides affect earthworms while exposed. As stated, the cucurbitacin-containing phytonematicides are allelochemicals or secondary metabolites that are lethal to nematodes, the

bioavailability of the products or chemical residues in produce would be evaluated to avoid food poisoning to human beings (Dube, 2016; Shadung *et al.*, 2017). The Vero cell lines would be suitable for cytotoxicity, while the microsome cells would be used for genotoxicity tests on cucurbitacin-containing phytonematicides (Ndhlala *et al.*, 2010). Results from Vero cell lines and microsome cells would help us understand how human cells exposed to the residues of the products would react. Moreover, Tetrazolium dye (MTT) assays would also be used to check how the human cells react when subjected to different amounts of Nemafric-BL and Nemarioc-AL phytonematicides.

To circumvent the issues of phytotoxicity, a computer-algorithm model called the Curve-fitting Allelochemical Response Dose (CARD) would be utilized to compartmentalize allelochemicals in living organisms (Liu *et al.*, 2003; Mashela *et al.*, 2021). According to Mashela *et al.*, (2017), allelochemicals affect the living entities through density-dependent growth (DDG) patterns, which have three phases: stimulation, neutral, and inhibition. The stimulant effect on plant growth was a non-problem as an agricultural product in plant protection, however, inhibition was a major concern and has been extensively examined in many crops (Bango *et al.*, 2019; Malebe, 2019; Sithole *et al.*, 2016). As a result, during pre-clinical registration of lead chemical compounds such as cucurbitacins, registration authorities would want assurances that the chemicals would not have cancerous, mutagenic, or cytotoxic effects on human cells and would not affect non-target organisms.

1.4 Purpose a study

1.4.1 Aim

Investigation of toxicity and bioavailability of cucurbitacin-containing phytonematicides on earthworms, selected model cells, and tomato plants.

1.4.2 Objectives

The objectives of the study were to:

- i. investigate the bioavailability of Nemarioc-AL and Nemafric-BL phytonematicides in plant tissues and chemical residues in different soil types.
- ii. examine whether exposure of earthworms to Nemafric-BL or Nemarioc-AL phytonematicide would affect earthworm protein content.
- iii. determine whether exposure to Raw 264.7 macrophage cells of Nemarioc-AL and Nemafric-BL phytonematicides would have toxic effects.
- iv. investigate whether short-term dermal and oral exposure to Nemarioc-AL and Nemafric-BL phytonematicides would induce mortality or any structural deformities on earthworms.

1.4.3 Null hypotheses

The null hypotheses of the study would:

- i. Nemarioc-AL and Nemafric-BL phytonematicides would not be bioavailable to the plant tissues and there would not be chemical residues in different soil types.
- ii. exposure of earthworms to Nemarioc-AL and Nemafric-BL phytonematicides would not affect earthworm protein content.

- iii. exposure of Raw 264.7 macrophage cells to Nemarioc-AL and Nemafric-BL phytonematicides would not have toxic effects.
- iv. short-term dermal and oral exposure of earthworms to Nemarioc-AL and Nemafric-BL phytonematicides would not result in mortality or any structural deformities.

1.5 Reliability, validity and objectivity

The statistical analysis of data at the 5% probability level served as the reliability for the current study. Validity was attained on time, whereas objectivity was attained by making sure that the results were presented based on evidence to remove subjectivity (Leedy and Ormrod, 2005).

1.6 Bias

By reducing the experimental error in every trial through replications, bias was kept to a minimum. Within the chosen experimental design, the treatments were randomized to ensure representative samples and increase precision of results (Leedy and Ormrod, 2005).

1.7 Scientific significance of the study

The CARD-computer based model, MTT and proteomics tests would resolve the problem of phytotoxicity on cucurbitacin-containing phytonematicides by providing the suitable non-phytotoxic concentrations for cucurbitacin-containing phytonematicides. The findings would make cucurbitacin-containing phytonematicides to be registered in the agrochemical market according to South African Act 36 of 1947.

1.8 Structure of the thesis

In the subsequent research chapter, the study investigates the null hypothesis formulated as :1. Nemarioc-AL and Nemafric-BL phytonematicides would not be bioavailable to the plant tissues and there would not be chemical residues in different soil types. 2. Exposure of earthworms to Nemarioc-AL and Nemafric-BL phytonematicides would not affect earthworm protein content. 3. exposure of Raw 264.7 macrophage cells to Nemarioc-AL and Nemafric-BL phytonematicides would not have cytotoxic effects. 4. Short-term dermal and oral exposure of earthworms to Nemarioc-AL and Nemafric-BL phytonematicides would not result in mortality or any structural deformities.

1.9 Ethical considerations

The usage of biopesticides, which were first introduced in Limpopo by the University of Limpopo, is envisaged in this study. The researcher would make sure that any prospective plaintiffs' legal or moral rights were upheld by the University. The ethical issues, suitable legal framework, and university regulations described here will remain in effect when the study is over.

1.10 References

- AL-Ahmadi, M.S. 2019. Pesticides, anthropogenic activities, and the health of our environment safety. In *Pesticides-Use and Misuse and Their Impact in the Environment*. IntechOpen.
- Alavanja, M.C., 2009. Introduction: Pesticides use and exposure, extensive worldwide. *Reviews on environmental health* 24:303-310.

- Alengebaw, A., Abdelkhalek, S.T., Qureshi, S.R. and W.Q Wang. 2021. Heavy metals and pesticides toxicity in agricultural soil and plants: Ecological risks and human health implications. *Toxics* 9:1-33.
- Ali, S., Ullah, M.I., Sajjad, A., Shakeel, Q. and A. Hussain. 2021. Environmental and health effects of pesticide residues. In *Sustainable Agriculture Reviews*, Vol 48, ed. Inamuddin, M. I. Ahamed and E. Lichtfouse, pp. 311–66 Cham, Switzerland: Springer.
- Ayilara, M.S., Adeleke, B.S., Akinola, S.A., Fayose, C.A., Adeyemi, U.T., Gbadegesin, L.A., Omole, R.K., Johnson, R.M., Uthman, Q.O. and O.O. Babalola. 2023. Biopesticides as a promising alternative to synthetic pesticides: A case for microbial pesticides, phytopesticides, and nanobiopesticides. *Frontiers in Microbiology* 14:1-16
- Bango, H., Shadung, K.G. and P.W. Mashela, 2019. Influence of Nemarioc-AL and Nemafric-BL phytonematicides on mineral nutrient elements in tomato leaf tissues under field conditions. *Research on crops*. 20:590-595.
- Datta, S., Singh, J., Singh, S. and J. Singh. 2016. Earthworms, pesticides and sustainable agriculture: a review. *Environmental Science and Pollution Research*. 23:8227-8243.
- Dube, Z.P. 2016. Nemarioc-AL and Nemafric-BL phytonematicides: Bioactivities in *Meloidogyne incognita*, tomato crop, soil type and organic matter. PhD Thesis, University of Limpopo, Sovenga, South Africa.
- Falya, Y., Sumiwi, S.A. and J. Levita. 2020. Mini review: Toxicity Study of Plant Extracts. *Journal of Pharmacy and Biological Sciences* 15:25-32.
- Ferreira, M.R.A., Daniele-Silva, A., De Almeida, L.F., Dos Santos, E.C.F., Machado, J.C.B., De Oliveira, A.M., Pedrosa, M.D.F.F., Paiva, P.M.G., Napoleão, T.H. and

- L.A.L Soares, 2022. Safety evaluation of aqueous extract from *Eugenia uniflora* leaves: Acute and subacute toxicity and genotoxicity in vivo assays. *Journal of Ethnopharmacology* 298:115668.
- Jansirani, D., Nivethitha, S. and M.V.P. Singh. 2012. Production and utilization of vermicast using organic wastes and its impact on *Trigonella foenum* and *Phaseolus aureus*. *Journal of Research in Biological Sciences International* 2:187-189.
- Joubert, P. and B. Sebata. 1982. The role of prospective epidemiology in the establishment of a toxicology service for a developing community. *South African Medical Journal* 62:853-854.
- Leedy, P.D. and J.E. Ormrod. 2005. Practical Research: Planning and Design. Upper Saddle River: NJ, Prentice Hall.
- Liu, D.L., Johnson, I.R. and J.V. Lovett. 2003. Mathematical modelling of allelopathy. III. A model for Curve-fitting Allelochemical Dose responses. *Non- Linearity in Biology, Toxicology and Medicine* 1:37-50.
- Malebe, A.L. 2019. Potential cucurbitacin chemical residues and non-phytotoxic concentration of two phytonematicide formulations in nightshade. Mini-dissertation, University of Limpopo, Sovenga, South Africa.
- Malherbe, S. and D. Marais. 2015. Economics, yield and ecology: a case study from the South African tomato industry. *Outlook on Agriculture* 44:37-47.
- Mashela, P.W. Shokoohi, E. and K.M. Pofu. 2020a. Morphological adjustments to hydrostatic pressure in pseudocoelomic cavity of *Steinernema feltiae* in response to Nemafric-BL phytonematicide. PLOS ONE, 1-14.
- Mashela, P.W., De Waele, D., Dube, Z., Khosa, M.C., Pofu, K.M., Tefu, G., Daneel, M.S. and H. Fourie. 2017. Alternative Nematode Management Strategies. In:

- Fourie, H., Spaull, V.W., Jones, R.K., Daneel, M.S. and D. De Waele (eds.). Nematology in South Africa: A View from the 21st Century. Springer International Publishers: Heidelberg.
- Mashela, P.W., Shimelis, H.A. and F.N. Mudau 2008. Comparison of the efficacy of ground wild cucumber fruits, aldicarb and fenamiphos on suppression of *Meloidogyne incognita* in tomato. *Journal of Phytopathology* 156:264-267.
- Mashela, S.T., Shadung, K.G., Mafeo, T.P. and P.W. Mashela. 2021. Non-phytotoxic concentration of cucurbitacin containing phytonematicides on Swiss chard (*Beta vulgaris* var. Cicla) under greenhouse conditions. *Research on Crops* 22:327-333.
- Morobe, I.C., Mthethwa, N.S., Bisi-Johnson, M.A., Vasaikar, S.D., Obi, C.L., Oyedeji, A.O., Kambizi, L., Eloff, J.N. and T. Hattori. 2012. Cytotoxic effects and safety profiles of extracts of active medicinal plants from South Africa. *Journal of Microbiology Research* 2:176-182.
- Mortelmans, K. and E. Zeiger. 2000. The Ames *Salmonella*/microsome mutagenicity assay. *Mutation research/fundamental and molecular mechanisms of mutagenesis* 455:29-60.
- Mphahlele, R.R., Mashela, P.W. and K.M. Pofu, 2012. Post-harvest fruit decay-inducing pathogen in medicinally important *Cucumis* species indigenous to South Africa. *African Journal of Agricultural Research* 6:3786-3791.
- Ndhlala, A.R., Anthonissen, R., Stafford, G.I., Finnie, J.F., Verschaeve, L. and J. Van Staden. 2010. *In vitro* cytotoxic and mutagenic evaluation of thirteen commercial herbal mixtures sold in KwaZulu-Natal, South Africa. *South African Journal of Botany* 76:132-138.

- Ndhlala, A.R., Ncube, B., Okem, A., Mulaudzi, R.B. and J. Van Staden. 2013. Toxicology of some important medicinal plants in southern Africa. *Food and chemical toxicology* 62:609-621.
- Ntlhamu, M.I., Ndhlala, A.R. and P. Masoko. 2021. Exploring the anti-HIV-1 reverse transcriptase, anti-inflammatory, anti-cancer activities and cytotoxicity of two fermented commercial herbal concoctions sold in Limpopo Province of South Africa. *BMC Complementary Medicine and Therapies* 21:1-12.
- Onkendi, E.M., Kariuki, G.M., Marais, M. and L.N. Moleleki. 2014. The threat of root-knot nematodes (*Meloidogyne* spp.) in Africa: A Review. *Plant Pathology* 63:727-737
- Pofu, K.M. and Mashela, P.W., 2022. Comparative efficacy of cucurbitacin phytonematicides and Velum on growth and fruit quality of watermelon cultivar 'Congo' and suppression of *Meloidogyne enterolobii* under field conditions. *Acta Agriculturae Scandinavica, Section B—Soil and Plant Science* 72:105-110.
- Seshweni, M.D. 2016. Integrated system for the management of *Meloidogyne javanica* in potato production. Mini-dissertation, University of Limpopo, Sovenga, South Africa.
- Shadung, K.G., P.W. Mashela, Mphosi, M.S. and V.L. Mulaudzi. 2017. Study of chemical residues from Nemarioc-AL and Nemafric-BL phytonematicides in tomato fruit. *African Journal of Agricultural Research* 12:1164-1168.
- Sikander, M., Hafeez, B.B., Malik, S., Alsayari, A., Halaweish, F.T., Yallapu, M.M., Chauhan, S.C. and M. Jaggi. 2016. Cucurbitacin D exhibits potent anti-cancer activity in cervical cancer. *Scientific reports* 6:1-13.
- Sithole N.T. 2021. Ameliorative effects of botanicals and rhizobacteria on the growth of *Pelargonium sidoides* and *Solanum lycopersicum* infested with *Meloidogyne*

incognita. PhD Thesis, University of KwaZulu-Natal, Pietermaritzburg, South Africa.

Sithole, N.T., Pofu, K.M., Mashela, P.W., Dube, Z.P. and H. Araya. 2016. Overall sensitivity of *Pelargonium sidoides* and root-knot nematodes to Nemarioc-AL phytonematicide. *Transylvanian Review* 11:2996-3001.

Van Wyk, A.S. and G. Prinsloo. 2020. Health, safety and quality concerns of plant-based traditional medicines and herbal remedies. *South African Journal of Botany* 133:54-62.

Yapıcı, S., Uraz, G. and E. Beyzi. 2021. A Study on the Possible Mutagenicity of Different Types of Plant Growth Regulators. *International Journal of Secondary Metabolite* 8:425-435.

CHAPTER 2

2. LITERATURE REVIEW

2.1 Introduction

The Cucurbitacins, which are tetracyclic triterpenes, are classified into groups, numbered A, B, C, D, E, F, G, H, I, J, K, L, O, P, Q, R and S, are mainly isolated from the Cucurbitaceae family (Delgado-Tiburcio *et al.*, 2022; Kaushik *et al.*, 2015; Varela *et al.*, 2022). The plant family Cucurbitaceae, sometimes known as cucurbits, cucurbitacins, or *Cucumis*, is renowned for its intensely bitter taste and possible health advantages counting anti-cancer antimalarial and antimicrobial activities (Varela *et al.*, 2022). This family includes about 800 species, around 130 genera, and less than 21 genera are commercialised and few wild species still under investigations (Rolnik and Olas, 2020.). As a result, Cucurbitaceae has been classified as a large family that includes several significant wild crops, including wild melon (*Cucumis africanus* L.), wild cucumber (*Cucumis myriocarpus* Naud.), and balsam apple (*Momordica balsamina* L.) (Maleka *et al.*, 2022). In South African rural communities, the fruits of wild melon and wild cucurcumber are used to treat liver damage, weakening of immune system, lumps, jaundice, inflammation and intestinal roundworm infection while the tender leaves are utilized as green leafy vegetable (Mphahlele *et al.*, 2012). Similar to balsam apple numerous ailments, such as diabetes, malaria, digestive problems, high blood pressure, and measles prevention, are treated with it (Thiaw *et al.*, 2023). The most common garden crops consumed from this family include but not limited to pumpkin (*Cucurbita moschata* L.), melon (*Cucumis melo* L.), watermelon (*Citrullus lanatus* Thunb.) and cucumber (*Cucumis sativus* L.) (Rajasree *et al.*, 2016). They are consumed as roasted seeds, cooked vegetables and fruit salads (Rezig *et al.*, 2019). The bioactive compounds from cucurbitacins have been demonstrated to be

concentrated on specific plant parts, such as the roots, leaves, and fruits (Kaushik *et al.*, 2015). A plethora of phytochemicals found in some wild Cucurbitaceae taxa may be harmful to people, animals, and soil ecosystems, which may discourage their usage for a variety of uses.

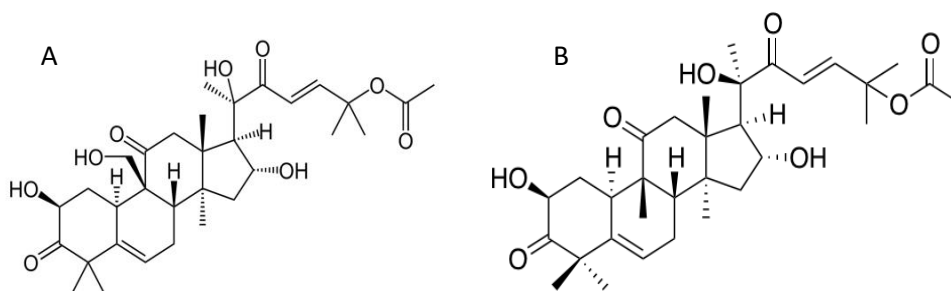


Figure 2.1 The chemical structures of cucurbitacin A ($C_{32}H_{46}O_9$) (A) and cucurbitacin B ($C_{32}H_{46}O_8$) (B) (Chen *et al.*, 2005, Shadung, 2016).

They have been shown to possess anticancer properties, anti-inflammatory, antimicrobial, antidiabetic and antioxidant activity (Kaushik *et al.*, 2015). Currently, the world is facing high occurrence of chronic diseases such as; cancer, chronic respiratory disease, diabetes and chronic cardiovascular diseases, just to mention a few are the major cause of mortality contributing 71% of premature death per annum (WHO, 2021). Of this 85% mortality happens globally, with the majority of cases falling within the age group of 30 to 69. In South Africa, premature death caused by chronic diseases is estimated at 51% annually, with about 26% belonging to the mentioned age group (WHO, 2019). In order to elude the exacerbating challenge of chronic diseases which results in premature death annually. Therefore, incorporation of cucurbitacin's in every day's diet has some promising impacts to mitigate mortality

caused by chronic diseases. Conversely, synthetic insecticides, nematicides, and fertilizers used in plant production are regarded as anthropogenic substances resulting from agricultural activities. They are toxic to non-target organisms or human beings with the possibility of causing cancer leading to premature mortality (Schmitt *et al.*, 2021). As a result, the crude extracts from cucurbitacin's have been recognized as alternative management strategy to synthetic nematicides (Mashela *et al.*, 2021).

The Cucurbitacins are the secondary metabolites produced by plants and are used as a defense mechanism against invasive pests or microorganisms (Rezig *et al.*, 2019). Moreover, cucurbitacin's are used in traditional medicines to treat diseases (Mphahlele *et al.*, 2012), and in pharmacology for drug development for cancer and other chronic ailments (Delgado-Tiburcio *et al.*, 2022). These Cucurbitacin's are the allelochemicals with the ability to cause toxicity to other living entities (Chen *et al.*, 2005; Mashela *et al.*, 2017). The bitter taste from Cucurbitaceae family is characterized as the cucurbitacins. Generally, the amount of cucurbitacins in Cucurbitaceae family differs from each other, based on their species types (Kaushik *et al.*, 2015). Most species that grow from the wild have a high amount of cucurbitacin in fruits such as wild melon, wild cucumber and *Momordica Balsamina* (Kaushik *et al.*, 2015). Although, wild melon has been shown to possess a high amount of Cucurbitacins throughout its organs. In cultivated cucumis, high concentrations of Cucurbitacins were detected from the roots, while low accumulations were found in fruits and varying based on variety or species type (Chen *et al.*, 2005). Therefore, the high concentrations of cucurbitacin's either in fruits, roots and leaves can induce toxicity to living entities as mentioned above. Efficacy trials need to be conducted to assess possible toxicity to ensure the overall safety on cucurbitacin's consumption.

Contrary, animal feed incorporated with wild cucurbitacin plant material has resulted in mortality on chickens, sheep's and goats while fed over a period (Gray, 2006). They are also intoxicated of cucurbitacins reported on human beings by zucchini squash (Gray, 2006). In addition, the withdrawal of synthetic chemical nematicides from the agrochemical market has resulted in the use of plant-based products as an alternative management strategy (Oka *et al.*, 2012). The wild cucurbitacins fruits are used for plant protection purposes to manage plant-parasitic nematodes (Mashela *et al.*, 2017). The efficacy of cucurbitacin-containing phytonematicides in nematodes suppression is well documented (Mashela *et al.*, 2008, Seshweni, 2016). Although, the cucurbitacin-containing phytonematicides have the potent to induce phytotoxicity on some protected cultigens. Phytotoxicity induced by cucurbitacins on cultigens was determined by using Curve-fitting Allelochemical Response Data (CARD) computer-based model (Sithole *et al.*, 2016). The objective of this review is to assess toxicity induced by cucurbitacins. Edible wild plants are used as a food source and a source of nutraceuticals and functional foods in many parts of the world.

2.2 Work done on problem statement

2.2.1 Bioavailability in produce from botanical remedies

The demand for quality and safe produce is high, since the withdrawal of non-fumigant nematicide increased, as shown by strict safety regulations on minimum chemical residues on imports on agricultural produce (Kumar *et al.*, 2019). Biopesticides were originally viewed as being eco-friendly, with non-toxic modes of actions (Senthil-Nathan, 2015) and at times, viewed as being safe without inducing chemical residues in produce (Kumar *et al.*, 2019; Senthil-Nathan, 2015). However, several studies as reviewed by Marín-Sáez *et al.* (2023) have reported the presence of persistent

bioactive substances in produce from plants protected from pests and diseases using biopesticides. The bioactive substances, technically known as secondary metabolites, are produced by plants during their interactions with biopesticides during defense (Rezig *et al.*, 2019). Since biopesticides affect living entities in different ways, it is important that the developed biopesticides be assessed for the accumulation of potential bioactive products, although in technical terms these are not chemical residues.

Three types of traditional methods commonly used to detect pesticide residues through instrumental techniques, include gas chromatography (GC), high-performance liquid chromatography (HPLC), and chromatographic methods coupled with mass spectrometry (MS) detectors. These techniques have become the most powerful analytical tools for determining polar organic compounds at $\mu\text{g/kg}$ or even ng/kg levels, offering the sensitivity, selectivity, and specificity required to comply with regulations (Masiá *et al.*, 2016). On peach (*Prunus persica*) trees treated with neem extracts, the accumulation of azadirachtin residues was not detected in both leaves and fruits at lower concentration levels but was detected at higher concentrations (Sarais *et al.* 2009). A study conducted on nightshade (*Solanum retroflexum*) plants treated by increasing concentrations of Nemarioc-AL, Nemafric-BL, Nemarioc-AG and Nemafric-BG phytonematicides, the cucurbitacin A and B were not detected on leaf material (Malebe, 2019). Similarly, Shadung (2016) did not detect chemical residues in tomato fruit after the plants were treated using the two test cucurbitacin-containing prototypes. However, to promote the safe use of biopesticides, both the European Union (EU) and the United States Environmental Protection Agency (USEPA) have implemented regulatory frameworks designed to protect human health, animals, and

the environment (Munjanja *et al.*, 2015). Maximum residue limits (MRLs) have been established globally, defining the highest amount of pesticide residue legally allowed in food (Munjanja *et al.*, 2015). Once the pesticide residues are shown to be present in produce, MRLs are determined by independent properly accredited laboratories.

2.2.2 Cytotoxicity effects of botanical remedies on cells

Cytotoxicity is the ability of a product to disrupt cellular functions and ultimately cause cell death (Funk *et al.*, 2022). Cells that are exposed to cytotoxic compounds may undergo necrosis (uncontrolled cell death) or apoptosis (programmed cell death) (Anil *et al.*, 2023). Various spectrophotometric methods are used to detect cytotoxicity, such as 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), neutral red uptake (NRU) and sulphorhodamine B (SRB) assay (Cordier and Steenkamp, 2015). In the current study MTT assay was used to test cytotoxicity of Nemarioc-AL and Nemafric-BL phytonematicides.

The MTT assay was first developed by R. T. Mosmann in 1983, and in terms of drug screening and cytotoxicity analysis, it is currently regarded as the golden standard method (Kus-Liśkiewicz *et al.*, 2021; Mosmann, 1983). This water-soluble yellow tetrazolium dye is made up of three aromatic rings, comprising two phenyl moieties and one thiazolyl ring, around a positively charged quaternary tetrazole ring core with four nitrogen atoms (Ghasemi *et al.*, 2021). In mitochondria of healthy cells, tetrazolium dye (yellow) is reduced by NADPH-dependent oxidoreductase enzymes in the cell cytosol and form purple formazan crystals (Boyraz *et al.*, 2021; Stefanowicz-Hajduk *et al.*, 2020). Then results would be analysed spectrophotometrically with IC₅₀

value mostly calculated. In a screening of cucurbitacin D, cucurbitacin E, and cucurbitacin I for toxicity using the MTT assay on two cancer cell lines, the observed IC_{50} values were 4.5, 6.8, and 3.8 μM in HT-29 cells, and 35, 19, 17, and 5 μM in the SW-480 cell line, respectively (Aftabi *et al.*, 2019). Generally, cucurbitacin I was more toxic against HT-29 cells and SW-480 cell lines than Cucurbitacin D and cucurbitacin E, with IC_{50} values of 3.8 and 17.5 μM , respectively (Aftabi *et al.*, 2019). In another study, the increasing concentrations of cucurbitacin-B against MDA-MB-231, SKBR3, MCF-7, and 4T-1 breast cancer cells reduced its survival in a time- and a concentration-dependent manner, with IC_{50} values ranging from 18 to 50 nM after 48–72 hours of treatment, with no effects on normal cells (Gupta and Srivastava, 2014). Therefore, the cytotoxicity of cucurbitacin-containing phytonematicides on living entities can be evaluated by using the MTT assays.

2.2.3 Responses of earthworms to botanical remedies

Earthworms are fondly referred to as the 'ecosystem civil engineers' due to their role in altering the physical, chemical, and biological characteristics of the soil through their activities (Astaykina *et al.*, 2022; Jones *et al.*, 1994). The earthworms directly or indirectly influence the availability of macro- and micronutrient elements essential for plants uptake and therefore, maintain soil productivity (Datta *et al.*, 2016). These organisms are believed to be the most important soil-enhancer in the soil ecosystem (Datta *et al.*, 2016). The abundance of earthworms on cultivated soil could result in a 25% yield increase and at least 23% increase in aboveground biomass (Van Groenigen *et al.*, 2014; Wang *et al.*, 2018). Earthworms are divided into three broad categories, namely, epigeic, endogeic and anecic based on their feeding preferences, burrowing abilities and size (Schelfhout, 2017). Epigeic earthworms are small bodied

and range from 1-7 cm in length, some are coloured bright red, although most have a reddish-brown skin pigmentation, which turn darker on their back and lighter on their tail and abdomen, for protection from ultra violet rays (Schelfhout, 2017). Endogeic earthworms are shallow-burrowing earthworms and live in and feed on the soil, making horizontal burrows through the soil to move around and to feed and would usually reuse the burrows, but to a certain extent (Schelfhout, 2017). Such earthworms are often exhibit colours that include grey, pale pink, green or blue. Finally, Anecic earthworms are deep-burrowing earthworms that make permanent vertical burrows in soil, feed on leaves in the soil surface that they drag into their burrows. Also, these earthworms cast (defecate) on the surface, and their casts can often be seen in grasslands (Schelfhout, 2017).

Intensification of plant protection practices to manage nematode population densities often result in a loss of non-targeted organisms, particularly the earthworms, as observed during the use of both fumigant and non-fumigant synthetic chemical nematicides (Hole *et al.* 2005; Pelosi *et al.*, 2014). Thus, the registration authorities require stringent ecotoxicology tests on earthworms.

The increasing concentration of algal extracts of *Spirulina platensis* A. as a developed biopesticide for plant protection were exposed to the earthworm, *Eisenia fetida* Sav. for 14 days in artificial soil under laboratory conditions, with the product declared safe since mortality on earthworms was not detected (Mohan *et al.*, 2019). In contrast, increasing concentration of neem (*Azadirachta indica* A. Jussieu.) leaf extract applied

at 0.50, 1.0, 2.0, 4.0 and 8.0 ppm against *Pheretima posthuma* (L. Vaillant) earthworms induced mortality rate of 10, 20, 30, 40 and 60%, respectively (Hussain *et al.*, 2017).

The test cucurbitacin-containing phytonematicides were shown to be highly toxic to r-nematode strategists even at low concentrations (Madaure *et al.*, 2017; Mashela *et al.*, 2016; Mashela *et al.*, 2024). Generally, r-nematode strategists are small in size, have high reproductive rates, shorter life cycles, and are very sensitive to environmental disturbances (Semprucci *et al.*, 2018). In contrast, the products were not toxic to K- nematode strategists that are large in size, with low reproduction rates, longer life cycles and quite tolerant to environmental disturbances. A good example of K-nematode strategists is an entomopathogenic nematode *Steinernema feltiae* (str. Mexican) which was shown to be tolerant to the test products (Madaure *et al.*, 2017). Additionally, Mashela *et al.*, (2024) indicated that the cucurbitacin-containing phytonematicides were not toxic to insects, fungi and bacteria.

Cucurbitacins constitute a group of diverse tetracyclic triterpenoid substances that are highly oxygenated, containing a cucurbitane skeleton characterized as 9 β -methyl-19-nor lanosta-5-ene (Díaz *et al.*, 2020). The cucurbitacin-containing phytonematicides are lipophilic and can disrupt the integrity of cell membrane of living entities if exposed (Mashela *et al.*, 2024). The effect of the products on nematodes is nematocidal as opposed to being nematostatic. Generally, when *Meloidogyne* species were exposed to cucurbitacin-containing phytonematicides, the products induced paralysis, which was irreversible (Dube, 2016; Mashela *et al.*, 2024). The mechanism was shown to involve the destruction of the cuticle (Mashela *et al.*, 2024). The cuticle of a nematode

is a complex structure that serves several functions, including protecting the organism from its environment and acting as an exoskeleton to which muscles are attached, thereby enabling movement and locomotion (Davies and Curtis, 2011). The cuticle is covered by a thin lipid layer, followed by five protein layers, comprising the basal, middle, and cortical layers (Page and Johnstone, 2007). The sorption of cucurbitacins can be attributed to the presence of functional groups in cucurbitacins that interact with lipids and subsequently hydrolyse the protein layer (Mashela *et al.*, 2024). Mashela *et al.* (2024) demonstrated that when *M. incognita* population densities was exposed to increasing concentration of Nemafric-BL phytonematicide total proteins versus the concentration exhibited negative quadratic relation, with breakdown of total proteins to a point referred to as isoprenylation to a minimum point. However, after the minimum point, synthesis of total proteins was observed.

In contrast to cuticles of nematodes, the body of earthworm is covered by a noncellular layer, which is technically called the epidermis, which does not specifically repel chemical substances but could play some important role to enhance tolerance of the earthworms to harmful substances (Zhou *et al.*, 2021). The epidermal mucus together with coelomic fluid discharged from the dorsal pores of earthworm form a mucus layer over their body surface, which has the potential to decrease the absorption of lethal pesticides (Heredia *et al.*, 2008; Zhou *et al.*, 2021). Generally, the mucus aids in maintaining the body moisture, preventing the drying up of the body which could be caused by lethal substances. Gudeta *et al.* (2023) demonstrated that the earthworm (*Annelid Oligochaeta*) had the ability to detoxify oxidative substances such as phenanthrene, azoxystrobin, plant polyphenols, and other toxic substances. Additionally, earthworms have the ability to mitigate the oxidative effects of

polyphenols through several adaptive mechanisms, which include the presence of unique metabolites, such as dialkylfuransulfonates (known as drilodefensins), which neutralise the oxidative impact of polyphenolic compounds secreted by plant material (Gudeta *et al.*, 2023). However, the review could not yield any information on how triterpenoids like cucurbitacins affect earthworms, except for *Azadirachta indica*, which has azadirachtin as main active ingredient and the others such as nimbolinin, nimbin, nimbidin, nimbidol, sodium nimbinate, gedunin, salannin, and quercetin (Alzohairy, 2016).

2.2.4 Assessing toxicity on earthworms

The long-used synthetic chemical pesticides are still utilized in plant production to control the challenge of pests and diseases that reduce yield and quality of agricultural produce for human consumption (AL-Ahmadi, 2019). Commercial farmers relied on this practice to maximize yield and to feed the growing population, with a lack of information on the toxicity induced by those products on non-target organisms (AL-Ahmadi, 2019). However, several studies reported that pesticides are harmful to the environment and have a negative impact on non-target species such as earthworms (Datta *et al.*, 2016; Pelosi *et al.*, 2021). In contrast, the use of synthetic chemical pesticides in plant production has increased compared to biopesticides. Despite strict regulations employed by the agrochemical market to reduce pesticide distribution to the environment (Pelosi *et al.*, 2021). Pesticides are distributed in to the environment during pesticide application, spray drift, chemical residues in produce volatilization, infiltration, and runoff from treated regions (Pelosi *et al.*, 2021). Necessitating to look further on how agricultural products affect non-target organisms when exposed.

Earthworm populations in agro-ecosystems are often very sensitive to the physico-chemical characteristics of the soil, which either directly or indirectly affect the nutrients available for earthworm survival (Singh *et al.*, 2020). Exposure of synthetic chemical pesticides have a negative effect on mortality or structural deformation on earthworms (Yuguda *et al.*, 2015). During pest control, pesticides, fungicides, herbicides, or nematicides applied to plants, bioaccumulate through their skin causing mortalities and structural deformities (Miglani and Bisht, 2019). These pesticides used in plant protection applied lead to morphological, behavioural, and physiological alterations in the reproductive, nervous, respiratory, and osmoregulatory systems of many soil organisms (Miglani and Bisht, 2019). These chemicals also contaminate the soil, resulting in detrimental effects on various invertebrates. Exposure of pyrethroid, neonicotinoids were highly toxic to earthworm (*Iumbricus terrestris*) under *In vitro* conditions inducing mortality and structural deformities (Yuguda *et al.*, 2015). Earthworm (*Eudrilus eugeniae*) treated by malathion pesticide structural deformities was observed such as coiling, and curling (Jeyaprakasam *et al.*, 2021). When 1,4-naphthoquinone (1,4-NTQ), a natural product developed from natural-origin walnut husk with nematocidal activities was tested against *Eisenia andre*, the product was sensitive to a test species (Chelinho *et al.*, 2017). However, they is a lack of information on how earthworms would respond to Nemarioc-AL and Nemafric-BL phytonematicide under *In vitro* conditions.

2.3 Work not done on research problem

The cucurbitacin-containing phytonemaicides are highly potent biopesticides for the management of plant nematode population densities. However, information on cytotoxicity to Vero cells and earthworms along with genotoxicity, bioavailability and

earthworms is undocumented. Thus, the current study was intended to close these perceived gaps in order to enable the UL research team to further consider the registration of Nemarioc-AL and Nemafric-BL phytonematicides with the South African registration authority as prescribed by Act 36 of 1947.

2.4 References

- Aftabi, Y., Zarredar, H., Sheikhi, M., Khoshkam, Z. and A. Hosseinzadeh Colagar. 2019. Induction of AHR Gene Expression in Colorectal Cancer Cell Lines by Cucurbitacin D, E, and I. *Journal of Cell and Molecular Research* 10:67-75.
- AL-Ahmadi, M.S. 2019. Pesticides, anthropogenic activities, and the health of our environment safety. In *Pesticides-Use and Misuse and Their Impact in the Environment*. IntechOpen.
- Alzohairy, M.A., 2016. Therapeutics role of *Azadirachta indica* (Neem) and their active constituents in diseases prevention and treatment. *Evidence-Based Complementary and Alternative Medicine* 2016:.7382506.
- Anil, S.; Sweety, V.; Vikas, B.; B. Joseph, 2023. Cytotoxicity and Cell Viability Assessment of Biomaterials. In *Cytotoxicity—Understanding Cellular Damage and Response*; IntechOpen: London, United Kingdom.
- Astaykina, A., Streletskii, R., Maslov, M., Krasnov, G. and V. Gorbatoov 2022. Effects of three pesticides on the earthworm *Lumbricus terrestris* gut microbiota. *Frontiers in microbiology* 13:1-12.
- Boyras, M.Ü., Shekhany, B. and F. Süzergöz, 2021. Cellular Imaging Analysis of MTT Assay Based on Tetrazolium Reduction. *Harran Üniversitesi Tıp Fakültesi Dergisi* 18:95-99.

- Chelinho, S., Maleita, C.M.N., Francisco, R., Braga, M.E.M., Da Cunha, M.J.M., Abrantes, I., De Sousa, H.C., Morais, P.V. and J.P. Sousa. 2017. Toxicity of the bionematicide 1, 4-naphthoquinone on non-target soil organisms. *Chemosphere* 181:79-588.
- Chen, J.C., Chiu, M.H., Nie, R.L., Cordell, G.A. and S.X. Qiu. 2005. The cucurbitacins and cucurbitane glycosides: Structures and biological activities. *Natural Products Report* 22:386-399.
- Cordier, W. and V. Steenkamp, 2015. Evaluation of four assays to determine cytotoxicity of selected crude medicinal plant extracts *in vitro*. *British Journal of Pharmaceutical Research* 7:16-21.
- Datta, S., Singh, J., Singh, S. and J. Singh. 2016. Earthworms, pesticides and sustainable agriculture: a review. *Environmental Science and Pollution Research* 23:8227-8243.
- Davies, K.G. and R.H. Curtis. 2011. Cuticle surface coat of plant-parasitic nematodes. *Annual review of phytopathology* 49:35-156.
- Delgado-Tiburcio, E.E., Cadena-Iñiguez, J., Santiago-Osorio, E., Ruiz-Posadas, L.D.M., Castillo-Juárez, I., Aguiñiga-Sánchez, I. and M. Soto-Hernández. 2022. Pharmacokinetics and biological activity of cucurbitacins. *Pharmaceuticals* 15:1-24.
- Díaz, M.T.B., Font, R., Gómez, P. and M.D.R. Celestino, 2020. Summer squash. In Nutritional composition and antioxidant properties of fruits and vegetables. *Academic Press*. 239-254.

- Dube, Z.P. 2016. Nemarioc-AL and Nemafric-BL phytonematicides: Bioactivities in *Meloidogyne incognita*, tomato crop, soil type and organic matter. PhD Thesis, University of Limpopo, Sovenga, South Africa.
- Funk, C.R., Wang, S., Chen, K.Z., Waller, A., Sharma, A., Edgar, C.L., Gupta, V.A., Chandrakasan, S., Zoine, J.T., Fedanov, A. and S.S. Raikar. 2022. PI3K δ/γ inhibition promotes human CART cell epigenetic and metabolic reprogramming to enhance antitumor cytotoxicity. *Blood, The Journal of the American Society of Hematology* 139:523-537.
- Ghasemi, M., Turnbull, T., Sebastian, S. and Kempson, I., 2021. The MTT assay: utility, limitations, pitfalls, and interpretation in bulk and single-cell analysis. *International Journal of Molecular Sciences*, 22:1-30.
- Gry, J., 2006. *Cucurbitacins in plant food*. Nordic Council of Ministers. Copenhagen.
- Gudeta, K., Kumar, V., Bhagat, A., Julka, J.M., Bhat, S.A., Ameen, F., Qadri, H., Singh, S. and R. Amarowicz, 2023. Ecological adaptation of earthworms for coping with plant polyphenols, heavy metals, and microplastics in the soil: A review. *Heliyon* 9:1-13.
- Gupta, P. and S.K. Srivastava. 2014. Inhibition of HER2-integrin signaling by Cucurbitacin B leads to *in vitro* and *in vivo* breast tumor growth suppression. *Oncology and Research Journal* 5:1812-1826.
- Heredía, R.B., Dueñas, S., Castillo, L., Ventura, J.J., Briano, M.S., Del Rio, F.P. and M.G. Rodríguez, 2008. Autofluorescence as a tool to study mucus secretion in *Eisenia foetida*. *Comparative Biochemistry and Physiology Part A: Molecular and Integrative Physiology* 151:407-414.

- Hole, D.G., Perkins, A.J., Wilson, J.D., Alexander, I.H., Grice, P.V. and A.D. Evans, 2005. Does organic farming benefit biodiversity. *Biological conservation* 122:113-130.
- Hussain, A., Khan, M.F., Faheem, M. and H. Rana, 2017. Toxicity of nitenpyram and neem leaf extract against earthworm. *International Journal of Biotechnology Research* 13:581-585.
- Jeyaprakasam, A., Muniyandi, B., James, A.J.P., Karmegam, N. and K. Ponnuchamy. 2021. Assessment of earthworm diversity and pesticide toxicity in *Eudrilus eugeniae*. *Environmental Chemistry and Ecotoxicology* 3:23-30.
- Jones, C. G., Lawton, J. H., and M. Shachak, 1994. Organisms as ecosystem engineers. *Oikos* 69:373. doi: 10.2307/354585.
- Kaushik, U., Aeri, V. and S.R. Mir. 2015. Cucurbitacins—an insight into medicinal leads from nature. *Pharmacognosy reviews* 9:12-18.
- Kumar, M., Kumar, S. and K. Kumar, 2019. Role of bio-pesticide in vegetables pest management: A review. *Journal of Pharmacognosy and Phytochemistry* 8:757-1763.
- Kus-Liśkiewicz, M., Fickers, P. and I Ben Tahar, 2021. Biocompatibility and cytotoxicity of gold nanoparticles: recent advances in methodologies and regulations. *International journal of molecular sciences* 22:1-21.
- Madaure, J. T., Mashela, P.W. and D. D. Waele. 2017. Responses of entomopathogenic nematode, *Steinernema feltiae* to cucurbitacin-containing Nemafric-BL phytonematicide. *Research on Crops* 18: 739-744.
- Malebe, A.L. 2019. Potential cucurbitacin chemical residues and non-phytotoxic concentration of two phytonematicide formulations in nightshade. Mini-dissertation, University of Limpopo, Sovenga, South Africa.

- Maleka, K.G., Mashela, P.W. and Pofu, K.M., 2022. Interactive effects of drenched triterpenoid phytonematicides on sugar content and plant growth of sweet sorghum (*Sorghum bicolor*) under microplot conditions. *Research on Crops* 23:562-565.
- Marín-Sáez, J., López-Ruiz, R., Romero-Gonzalez, R. and A. Garrido Frenich. 2023. Comprehensive dissipation of azadirachtin in grapes and tomatoes: The effect of *Bacillus thuringiensis* and tentative identification of unknown metabolites. *Journal of Agricultural and Food Chemistry* 71:4466-4476.
- Mashela, P., Pofu, K.M. and A.R. Ndhlala. 2024. Nemafric-BL Phytonematicide Induces Isoprenylation and Farnesylation in Guava Root-Knot Nematode, *Meloidogyne enterolobii*: Mechanism of Cucurbitacin-Cuticle Interaction. doi:10.20944/preprints202403. 151.v1.
- Mashela, P.W., De Waele, D., Dube, Z., Khosa, M.C., Pofu, K.M., Tefu, G., Daneel, M.S. and H. Fourie. 2017. Alternative Nematode Management Strategies. In: Fourie, H., Spaull, V.W., Jones, R.K., Daneel, M.S. and D. De Waele (eds.). *Nematology in South Africa: A View from the 21st Century*. Springer International Publishers: Heidelberg.
- Mashela, P.W., Shimelis, H.A. and F.N. Mudau 2008. Comparison of the efficacy of ground wild cucumber fruits, aldicarb and fenamiphos on suppression of *Meloidogyne incognita* in tomato. *Journal of Phytopathology* 156:264-267.
- Mashela, P.W., Shimelis, H.A. and F.N. Mudau 2008. Comparison of the efficacy of ground wild cucumber fruits, aldicarb and fenamiphos on suppression of *Meloidogyne incognita* in tomato. *Journal of Phytopathology* 156:264-267.

- Mashela, S.T., Shadung, K.G., Mafeo, T.P. and Mashela, P.W., 2021. Non-phytotoxic concentration of cucurbitacin containing phytonematicides on Swiss chard (*Beta vulgaris* var. Cicla) under greenhouse conditions. *Research on Crops* 22:327-333.
- Masiá, A, M.M. Suarez-Varela, A. Llopis-Gonzalez, and Y. Picó 2016. "Determination of pesticides and veterinary drug residues in food by liquid chromatography-mass spectrometry: A review." *Analytica Chimica Acta* 936:40-61.
- Mohan, S.S., Hilda, S., Chandrasehar, G. and A. Goparaju. 2019. Effect of *Spirulina platensis* extracts on earthworm, *Eisenia fetida*. *Journal of Advanced Scientific Research* 10:130-136.
- Mosmann, T. 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *Journal of immunological methods* 65:55-63.
- Mphahlele R, Mashela P.W, K.M. Pofu. 2012. Post-harvest fruit decay-inducing pathogen in medicinally important *Cucumis* species indigenous to South Africa. *African Journal of Agricultural Research* 6:3786-3791.
- Munjanja, B., Chaparadza, A. and S. Majoni. 2015. Biopesticide residue in foodstuffs. *Biopesticides handbook*. CRC Press, Boca Raton, pp.71-92.
- Oka, Y., Ben-Daniel, B. and Y. Cohen. 2012. Nematicidal activity of the leaf powder and extracts of *Myrtus communis* against the root-knot nematode *Meloidogyne javanica*. *Plant Pathology* 61:1012-1020.
- Page, A. P., and I. L. Johnstone 2007. The cuticle, WormBook, ed. In WormBook. The *C.elegans* Research Community. doi/10.1895/wormbook.1.138.1<http://www.wormbook.org>.

- Pelosi, C., Barot, S., Capowiez, Y., Hedde, M. and F Vandebulcke. 2014. Pesticides and earthworms. A review. *Agronomy for Sustainable Development* 34:199-228.
- Pelosi, C., Bertrand, C., Daniele, G., Coeurdassier, M., Benoit, P., Nélieu, S., Lafay, F., Bretagnolle, V., Gaba, S., Vulliet, E. and C. Fritsch. 2021. Residues of currently used pesticides in soils and earthworms: A silent threat? *Agriculture, Ecosystems and Environment* 305:107167.
- Rajasree, R.S., Sibi, P.I., Francis, F. and H. William. 2016. Phytochemicals of Cucurbitaceae family-A review. *International Journal of Pharmacognosy and Phytochemical Research* 8:113-123.
- Rezig, L., Chouaibi, M., Meddeb, W., Msaada, K. and S. Hamdi. 2019. Chemical composition and bioactive compounds of Cucurbitaceae seeds: Potential sources for new trends of plant oils. *Process Safety and Environmental Protection* 127:73-81.
- Rolnik, A. and B. Olas. 2020. Vegetables from the Cucurbitaceae family and their products: Positive effect on human health. *Nutrition* 78:1-6.
- Sarais, G., Angioni, A., Lai, F., Cabras, P. and Caboni, P., 2009. Persistence of two neem formulations on peach leaves and fruit: effect of the distribution. *Journal of agricultural and food chemistry* 57:2457-2461.
- Schelfhout, S., Mertens, J., Verheyen, K., Vesterdal, L., Baeten, L., Muys, B. and A. De Schrijver. 2017. Tree species identity shapes earthworm communities. *Forests* 8:1-20.

- Schmitt, F., Babylon, L., Dieter, F. and G.P. Eckert. 2021. Effects of Pesticides on Longevity and Bioenergetics in Invertebrates—The Impact of Polyphenolic Metabolites. *International Journal of Molecular Sciences* 22:13478.
- Semprucci, F., Cesaroni, L., Guidi, L. and M. Balsamo. 2018. Do the morphological and functional traits of free-living marine nematodes mirror taxonomical diversity?. *Marine environmental research* 135:114-122.
- Senthil-Nathan, S. A. 2015. review of biopesticides and their mode of action against insect pests. *In Environmental Sustainability*; Springer: Berlin/Heidelberg, Germany 49–63.
- Seshweni, M.D. 2016. Integrated system for the management of *Meloidogyne javanica* in potato production. Mini-dissertation, University of Limpopo, Sovenga, South Africa.
- Shadung, K.G. 2016. Quality protocols for Nemarioc-AL and Nemafric-BL phytonematicides and potential chemical residues in tomato fruits. PhD Thesis, University of Limpopo, Sovenga, South Africa.
- Singh, S., Sharma, A., Khajuria, K., Singh, J. and A.P. Vig. 2020. Soil properties changes earthworm diversity indices in different agro-ecosystem. *BMC ecology* 20:1-14.
- Sithole, N.T., Pofu, K.M., Mashela, P.W., Dube, Z.P. and H. Araya. 2016. Overall sensitivity of *Pelargonium sidoides* and root-knot nematodes to Nemarioc-AL phytonematicide. *Transylvanian Review* 11:2996-3001.
- Stefanowicz-Hajduk, J. and Ochocka, 2020. Real-time cell analysis system in cytotoxicity applications: Usefulness and comparison with tetrazolium salt assays. *Toxicology reports* 7:335-344.

- Thiaw, M., Samb, I., Genva, M., Gaye, M.L. and M.L. Fauconnier, 2023. *Momordica balsamina* L.: A Plant with Multiple Therapeutic and Nutritional Potential—A Review. *Nutraceuticals*, 3:556-573.
- Varela, C., Melim, C., Neves, B.G., Sharifi-Rad, J., Calina, D., Mamurova, A. and C. Cabral, 2022. Cucurbitacins as potential anticancer agents: New insights on molecular mechanisms. *Journal of Translational Medicine* 20:630.
- Yuguda, A.U., Abubakar, Z.A., Jibo, A.U., AbdulHameed, A. and A.J. Nayaya. 2015. Assesment of toxicity of some agricultural pesticides on earthworm (*Lumbricus terrestris*). *American-Eurasian Journal of Sustainable Agriculture* 9:49-59.
- Zhou, X., Liang, W., Zhang, Y., Ren, Z. and Y. Xie, 2021. Effect of earthworm *Eisenia fetida* epidermal mucus on the vitality and pathogenicity of *Beauveria bassiana*. *Scientific Reports* 11:1-11.

CHAPTER 3

DETERMINING THE BIOAVAILABILITY OF CUCURBITACINS FROM CUCURBITACIN-CONTAINING PHYTONEMATOCIDES ON TOMATO PLANTS UNDER DIFFERENT SOIL TYPES

Abstract

The economic importance, phytochemical composition, and health benefits of tomato plant make it a significant fruit crop in our everyday lives. However, the plant is susceptible to many pests and diseases, including plant parasitic nematodes. Therefore, for significant harvest, synthetic pesticides must be used during the production process. Be that as it may, synthetic nematicides are deemed toxic, negatively affecting non-target organisms. This has led to the development of natural nematicides namely, Nemarioc-AL and nemafric-BL phytonematicides to manage plant parasitic nematodes. The objective of the study was to determine the accumulation of cucurbitacin residues in tomato plant parts on selected soil types after inoculation to control nematodes. Seedlings were transplanted into 20 cm plastic pots filled separately with different soil types (sandy loam, dark soil, red soil, silt soil, sandy soil and sandy loam (+)). Seven days after transplanting, the pots were divided into two groups. The first group was treated with Nemarioc-AL phytonematicide, while the second group was treated with Nemafric-BL phytonematicide at 3% each. After 150 days of applying phytonematicides, fruits were collected and analyzed for the accumulation of cucurbitacin residues in a Waters Acquity Classic ultra-performance liquid chromatograph connected to a Waters Xevo TQ-MS mass spectrometer. As a result, a much lower concentration of cucurbitacin accumulation was detected in produce. Both phytonematicides can be used for the intended purpose.

Keywords: Phytonematicide, Pesticide, nematodes, environment, bioavailability

3.1 Introduction

Around the world, plants are vital in human lives because they provide us with food, medicine, and industrial materials. They are also important to many cultures, religions, and medical practices (Schaal, 2019). Although, biotic stressors like pests and diseases as well as abiotic stressors like temperature changes, have a negative impact on plants (Azlay *et al.*, 2023). This could limit the amount of resources plants can provide to the world's rapidly growing population. Tomato (*Solanum lycopersicum* L..) plants are regarded as a good source of phytochemicals and nutrients such as iron, potassium and vitamin c (Collins *et al.*, 2022). Additionally, they are a source of antioxidants like as beta-carotene and phenolic compounds such as flavonoids, hydroxynnnamic acid, chlorogenic, homovanillic acid and ferulic acid (Collins *et al.*, 2022). However, a variety of pests can affect tomato plants, such as root-knot (*Meloidogyne* species) nematodes, which can lower yield both directly and indirectly (Onkendi, 2014). As a result, in plant protection, there is rampant use of pesticides like nematicides, insecticides, miticides, herbicides and fungicides to control pests.

The fate and transport of these compounds and their accumulation in plants is necessary for safeguarding human safety. According to Yu *et al.*, (2006), hydrophobic organic molecules are transported and ultimately disposed of in soils through intricate mechanisms that are subject to various processes such as volatilization, leaching/desorption, adsorption, and chemical or biological degradation in the environment. As a result, bioavailability is one of the important factors to consider after application of pesticides.

The percentage of a chemical present in the environment that may pass through cell membranes and be absorbed by living entities is known as bioavailability (Hodson *et al.*, 2010). Adsorption is the process by which the surfaces of soil colloids use ion exchange to retain solutes in the soil solution. Pesticide physical and chemical qualities (carbon rings, ionic form, etc.) as well as soil variables (soil texture, mineral composition, organic matter content, specific surface area, and soil solution chemistry) all have an impact on bioavailability (Kanissery *et al.*, 2019). Therefore, different soil types may be used to assess bioavailability of pesticides in tomato roots, leaves, stem and fruits.

Synthetic chemical pesticides like velum, aldicarb, nemacur, and fenamiphos were utilized to control nematodes (Seshweni *et al.*, 2016). Due to their environmental unfriendliness, they were withdrawn from the agrochemical in 2005, exacerbating the challenge of plant parasitic nematodes on crops (Mashela *et al.*, 2015). Therefore, natural pesticides like cucurbitacin-containing phytonematicides were developed as an alternative to synthetic chemical nematicides (Mashela *et al.*, 2017). Cucurbitacin-containing phytonematicides were developed from fruit extracts of the indigenous plants of South Africa, namely wild water melon (*Cucurmis myriocupus*) and wild cucumber (*Cucurmis africanus*) (Mashela *et al.*, 2017). As products designed for plant protection, they could be toxic and meant to control nematodes on a variety of crops. However, information of toxicity on bioactive compounds from stated phytonematicides in tomato fruits is required. Therefore, there is a need to assess the bioavailability in produce to ensure safety for non-target organisms. The objective of this study was to determine the bioavailability and chemical residues of Nemafric-BL or Nemarioc-AL phytonematicides in plant tissues under different soil types.

3.2 Materials and Methods

3.2.1 Description of the study location

The research was conducted at the Green Biotechnologies Research Centre of Excellence (GBRCE), University of Limpopo, South Africa (23°53'10"S, 29°44'15"E). The temperature in a greenhouse was kept between 20 and 21°C during the day and at night using thermostatically controlled fans on the north side and damp walls on the south facing side. The greenhouse's heterogeneous conditions are a result of wet walls and thermostatically activated fans creating wind-generated currents that drastically chill the air as it passes through the growth area. while selecting a research design inside a greenhouse because of the diverse conditions produced by the moist wall and thermostatically activated fans. This research was carried out during the spring of 2023 (March – August) and validated in 2024.

3.2.2 Treatments and Experimental Design

Two separate trials for Nemarioc-AL phytonematicide (3%) and Nemafric-BL phytonematicide (3%) were conducted separately on sandy loam, dark soil, red soil, silt soil, sandy soil, sandy loam (+) arranged in randomized complete block design (RCBD) and replicated 6 times. Blocking was done with regard to the varied conditions inside the greenhouse.

3.2.3 Procedures and Cultural Practices

Nemarioc-AL phytonematicide and Nemafric-BL phytonematicide were firstly prepared by using a locally developed method at the University of Limpopo, South Africa (Mashela *et al.*, 2017). In a nutshell, the Nemarioc-AL phytonematicide and Nemafric-BL phytonematicide production involved adding 16 L of chlorine-free tap water to a 20 L hermetically sealed plastic container. Approximately, 80 g and 40 g of dried and powdered *C. myriocopus* and *C. africanus* fruit were added to each container along with 300 mL of concentrate effective microorganisms (EM) (EMRO, Japan), 300 mL of molasses and 100 g of sugar. Then, the ingredients were added to the containers and shaken to combine. To allow the generated CO₂ from the containers to escape during the fermentation process, the containers had an outlet. This outlet was an airtight pipe with a 5-mm diameter and 50 cm length, with one end glued to a hole on the lid of the 20 L containers and the other end dangling into a 2-liter bottle half-filled with chlorine-free tap water. In order to allow the fermentation-induced pH to decrease by roughly 3.7 units, the airtight system was left at room temperature for 14 days (Kyan *et al.*, 1999).

Seed of tomato plants, cv. 'Floradate', were sown in a 200-hole seedling tray filled with Hygromix-T (Hygrotech, Pretoria North, South Africa), placed on a greenhouse bench and irrigated regularly. At the 4-leaf stage, seedlings were hardened-off by withholding irrigation under direct sunlight, with water supplied when 80% seedlings were showing some degree of wilting. The process was repeated for 7 days prior to returning the seedlings to the greenhouse for transplanting. Different soil types, sandy loam, dark soil, red soil, silt soil, sandy soil and sandy loam soil (+) were separately steam-pasteurised (300°C for 1 h) and kept overnight outside of the pasteurised pot to cool.

Each soil component was filled in 20-cm-diameter plastic pots with a total volume of 2,700 mL of growing mix. After transplanting uniform seedlings, pots were arranged on the greenhouse benches at a 0.6 × 0.6 m spacing and grouped into two sets for Nemarioc-AL phytonematicide and Nemafric-BL phytonematicide treatments. At transplanting, each plant was fertilized using 5 g of 2:3:2 (26) NPK and two weeks after transplanting 2 g of 2:1:2 (43) Multifeed (Nulandies, Johannesburg, South Africa) was applied. Irrigation was achieved by using 200 mL of chlorine-free water every other day. Monitoring for insect pests and diseases inside the greenhouse were carried out daily. Coragen and complete 350 pesticide were used to manage white flies (*Trialeurodes vaporariorum* Westwood.) and Tuta absoluta (*Phthorimaea absoluta* Meyrick.) as per label instructions. Basil (*Ocimum basilicum* L.) and Momordica (*Momordica balsamina* L) were used as repellent plants inside the greenhouse.

Soil pH and electrical conductivity (EC)

Soil pH and soil electrical conductivity (EC) were determined according to Okalebo *et al.* (2002). The method involves weighing 20 g of each soil sample and adding 50 mL of deionized water to a 100 mL tube. The mixture was stirred for 10 min using a shaker (labcon, Laboratory Marketing service cc., South Africa) and allowed to settle for 30 min and pH was measured (Multiparameter EC Meter edge, Hanna Instruments South Africa).

3.2.4 Data Collection

At 150 days after application of Nemarioc-AL and Nemafric-BL phytonematicides, shoots were cut off from the roots above the ground and weighed for fresh shoot and root mass. After that, they were dried in an oven at 52 °C for 72 hours and then ground in a Wiley mill.

3.2.5 Determination of residues and bioavailability in different plant parts

Approximately, 0.25 g each of dried and ground roots, stems, leaves and fruits were separately weighed out into a 15 mL falcon tube. Then extracted with 10 mL methanol with vortexing and standing overnight. Thereafter, each of the samples was diluted 5x with methanol after centrifugation for 7 min and transferred to vials. Liquid chromatographic separation of plasma samples (3 µL) was performed at 30 °C on a BEH C18 column (2.1 × 50 mm, 1.7 µm; Waters, Milford, United States), using a Waters Acquity Classic ultra-performance liquid chromatograph connected to a Waters Xevo TQ-MS mass spectrometer (Waters, Milford, MA, USA). The mobile phase consisted of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B) delivered at a flow rate of 0.4 mL/min in gradient mode.

3.2.5 Data Analysis

Data for concentration response curves were analysed by GraphPad Prism® version 5.2, GraphPad Software Inc., San Diego, CA, USA (Motulsky, 2007).

3.3 Results and discussion

3.3.1 Assessing cucurbitacin residues in tomato fruits

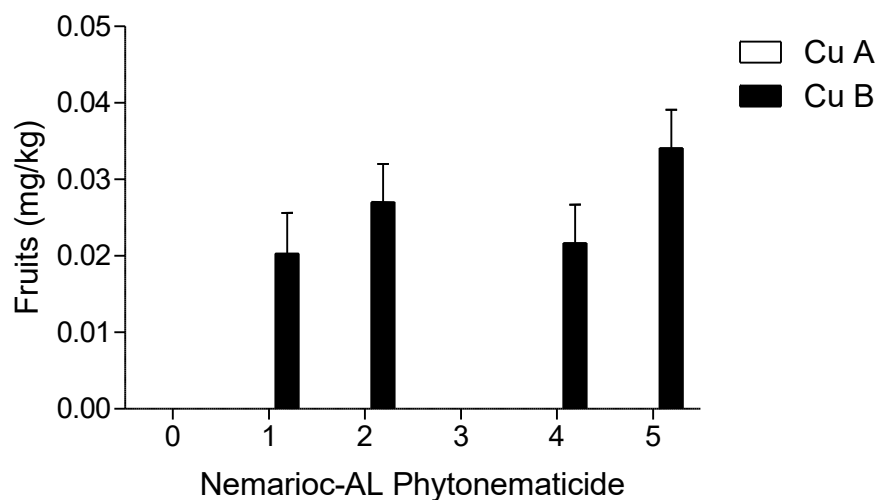


Fig 3.1. The effect of Nemarioc-AL phytonematicide on accumulation of cucurbitacin A and B residues in tomato fruits under sandy loam, dark soil, red soil, sandy soil, and sandy loam (+) (n=36). Cu A = cucurbitacin A; Cu B = cucurbitacin B.

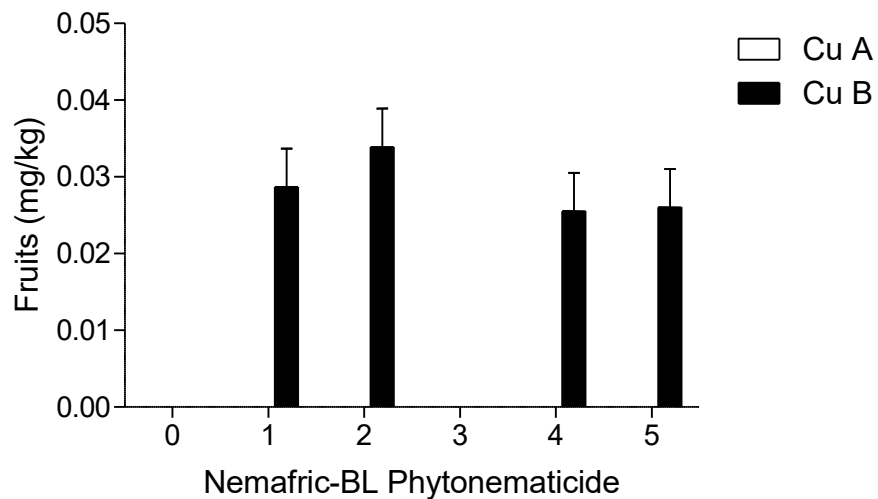


Fig 3.2. The effect of Nemafric-BL phytonematicide on accumulation of cucurbitacin A and B residues in tomato fruits under sandy loam, dark soil, red soil, sandy soil, and sandy loam (+) (n=36). Cu A = cucurbitacin A; Cu B = cucurbitacin B.

Accumulation of cucurbitacin A and B was assessed in tomato fruits harvested from sandy loam, dark soil, red soil, sandy soil, and sandy loam (+), while silt soil did not produce matured fruits fit to be analyzed. At 150 days after application of Nemarioc-AL phytonematicide, the accumulation of cucurbitacin A in tomato fruits was not detected, while cucurbitacin B was detected. Sandy loam (+), had the highest concentration of cucurbitacin B (0.034 mg/kg), followed by red soil with 0.027 mg/kg, then sandy soil with 0.022 mg/kg and red soil with 0.20 mg/kg. No accumulation of both cucurbitacin's was detected in the untreated control. In Nemafric-BL treated soil, accumulation of cucurbitacin A was not detected, while cucurbitacin B was detected. The highest cucurbitacin B accumulation was detected in red soil, followed by dark soil, then followed sandy soil and sandy loam (+) with an average mean of 0.034, 0.028, 0.025 and 0.026, respectively. Numerous enzymes found in plants are capable of breaking down substances that are given to them. It is probable that cucurbitacin A is being quickly transformed into cucurbitacin B in the tomato fruits. Plant enzymes (such cytochrome P450 monooxygenases or other oxidative enzymes) turn cucurbitacin A into cucurbitacin B in this biotransformation, which may be a natural detoxification or modifying process. As a result, despite the initial application of cucurbitacin A, only cucurbitacin B would accumulate and be detectable.

The stated results contradict findings by Shadung (2016), when Nemarioc-AL phytonematicide was applied on tomato plants cv. 'Rodade', cucurbitacin A and B were not detected in fruits. While in the current study cucurbitacin B was detected. In another study where Nemarioc-AL phytonematicide was applied on 'Floradade' they were no accumulation of cucurbitacin A and B residues in fruits (Bango, 2019). Malebe, (2019) reported no accumulation of cucurbitacin residues on night shade (*Solanum*

retroflexum Dun.) treated by Nemarioc-AL phytonematicide. But agree with the findings where neem essential oil (*Azadirachta indica* Juss.) was used as a bioinsecticide against sucking insect pests on eggplant (*Solanum melongena* L.) production, azadirachtin the active ingredients in neem was detected in fruits (Takla *et al.*, 2021). Non accumulation of cucurbitacin A in fruits, support the idea that this compound was partial polar molecule, meaning slightly soluble in water (Chen *et al.*, 2005).

3.3.2 Testing cucurbitacin residues in tomato leaves

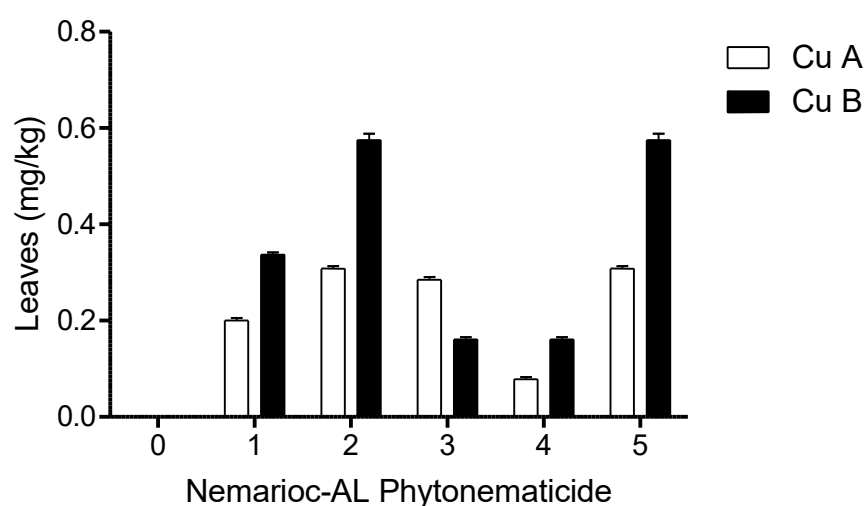


Fig 3.3. The effect of Nemafric-BL phytonematicide on accumulation of cucurbitacin A and B residues in tomato fruits under sandy loam, dark soil, red soil, sandy soil, and sandy loam (+) (n=36). Cu A = cucurbitacin A; Cu B = cucurbitacin B.

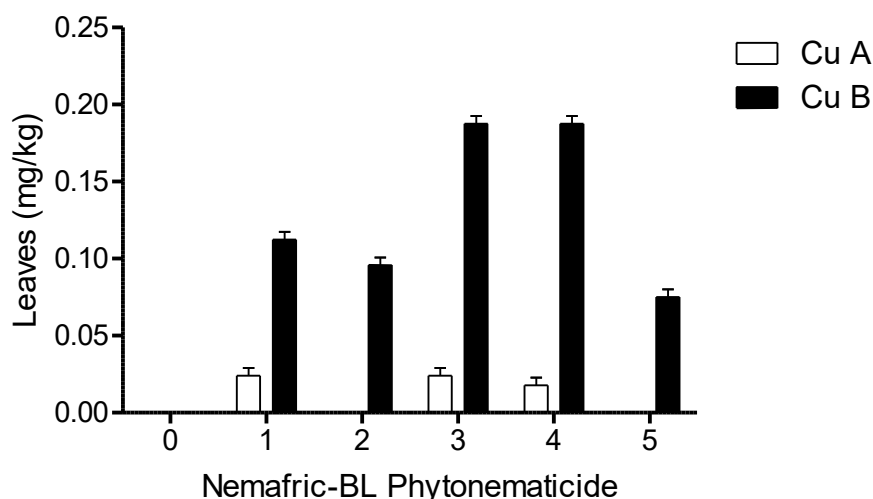


Fig 3.4. The effect of Nemafric-BL phytonematicide on accumulation of cucurbitacin A and B residues in tomato fruits under sandy loam, dark soil, red soil, sandy soil, and sandy loam (+) (n=36). Cu A = cucurbitacin A; Cu B = cucurbitacin B.

Nemarioc-AL phytonematicide-treated dark soil, red soil, silt soil, sandy soil and sandy loam (+), had an accumulation of cucurbitacin residues, while untreated sandy loam did not exhibit any cucurbitacin residues (Figure 3.3). Cucurbitacin A and cucurbitacin B were both detected in tomato leaves, with cucurbitacin B being the most prevalent than cucurbitacin A. The highest concentrations of the bioactive compound cucurbitacin A were detected in red, silt, and sandy loam soil (+), with 0.31, 0.28, and 0.31 mg/kg, respectively (Figure 3.3). Dark soil was next with an average of 0.31 mg/kg, and sandy soil had the lowest concentration, with an average of 0.08 mg/kg (Figure 3.3). In contrast, the highest accumulation of cucurbitacin B was in red and sandy loam soil (+), with an average mean of 0.57 mg/kg (Figure 3.3). Then secondly came dark soil with an average mean of 0.34 mg/kg; lastly, silt and sandy soils were the lowest with 0.16 mg/kg (Figure 3.3).

Generally, Nemarioc-AL phytonematicide was known to possess only cucurbitacin A, which is non polar molecule that partially dissolve in water and breaks down into leptodermin and curcumin (Chen *et al.*, 2003). In current study, more than one cucurbitacin was isolated. The cucurbitacin B was also detected predominantly with higher concentration level than cucurbitacin A. This shows the *C. myriocapus* fruits besides containing cucurbitacin A it also contains abundance of cucurbitacin B which was firstly detected in this study. This finding corroborates with findings by Shaik *et al.* (2017), where in *C. myriocapus* fruits cucurbitacin A and C were isolated and in roots and leaves cucurbitacin A and C were also detected (Grubben and Denton, 2004). The presence of cucurbitacin A and B in leaves in the current study contradicts with findings on tomato plants (Shadung, 2016). That states that, since the two cucurbitacin's are non-polar molecules cannot be transported through the bipolar membranes of the symplastic pathway of the endodermis in roots into the vascular bundle to various parts of a plant (Campbell, 1990; Shadung, 2016).

3.3.3 Detecting cucurbitacin residues in tomato stems

The highest cucurbitacin A residues on different soil types treated by Nemarioc-AL phytonematicide were detected from red and silt soil with the average mean of 0.33 mg/kg, then followed by sandy soil and sandy loam soil (+) with an average mean of 0.21 and 0.18 mg/kg, respectively (Figure 3.5). The least was dark soil with an average mean of 0.05 mg/kg. In contrast, the highest cucurbitacin B detected in tomato stems after application of Nemarioc-AL phytonematicide was dark soil and red soil with an average mean of 0,39 mg/kg, then followed by sandy loam soil with 0.22 mg/kg and lastly it was silt and sandy soil with an average mean of 0.18 mg/kg (Figure 3.5). The Nemafric-BL treated soil in tomato stems had higher concentration of cucurbitacin B

on sandy loam soil (+) with average mean of 3.44 mg/kg, followed by silt, sandy and red soil with the average mean of 0.16, 1.14 and 0.13 mg/kg, respectively (Figure 3.6). In contrast, Nemafric-BL phytonematicide treated soil had a smaller accumulation of cucurbitacin A, with red soil slightly higher with 0.07 mg/kg (Figure 3.6). Sandy loam soil (+) followed, and the least was sandy and silt soil with 0.029 and 0.024 mg/kg, respectively. Lastly red soil had no accumulation of cucurbitacin A (Figure 3.6).

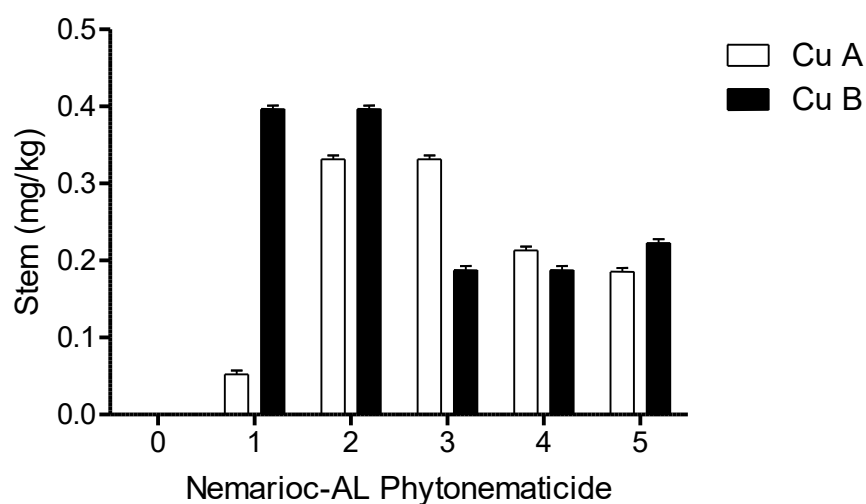


Fig 3.5. The effect of Nemarioc-AL phytonematicide on accumulation of cucurbitacin A and B residues in tomato stem under sandy loam, dark soil, red soil, sandy soil, and sandy loam (+) (n=36). Cu A = cucurbitacin A; Cu B = cucurbitacin B.

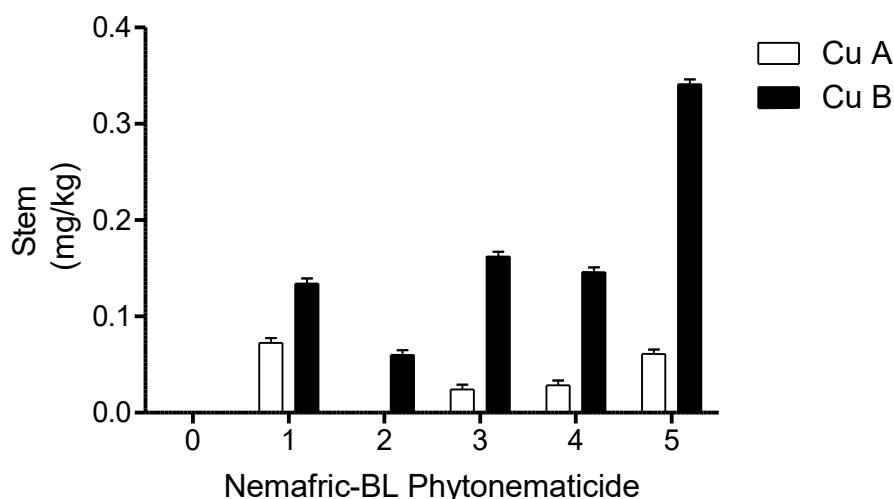


Fig 3.6. The effect of Nemarioc-AL phytonematicide on accumulation of cucurbitacin A and B residues in tomato stem under sandy loam, dark soil, red soil, sandy soil, and sandy loam (+) (n=36). Cu A = cucurbitacin A; Cu B = cucurbitacin B.

The accumulation of cucurbitacin residues on Nemarioc-AL phytonematicide treated soil have shown the presence of cucurbitacin A was comparable to that of cucurbitacin B. This indicates that Nemarioc-AL phytonematicide contains high amounts cucurbitacin A with smaller concentrations of cucurbitacin B. In contrast, Nemafric-BL phytonematicide treated soil had higher accumulation of cucurbitacin B as compared to cucurbitacin A. Nemarioc-AL phytonematicide is known to contain cucurbitacin A which is a bioactive compound that has nematicide properties (Mashela *et al.*, 2024). However, this study has identified cucurbitacin B bioactive compounds in tomato plant parts. At some other stages the cucurbitacin B was more dominant than cucurbitacin A. Dark and red soil have accumulated the highest cucurbitacin accumulation suggesting that heavy soil can retain moisture for a long time holding the phytonematicide so long increasing chances for absorption.

3.3.4 Cucurbitacin residues in tomato roots

The accumulation of cucurbitacin A residues in tomato plants roots treated by Nemarioc-AL phytonematicide was detected at higher concentration level on sandy soil at average mean of 0.16 mg/kg, followed by silt soil at 0.08 mg/kg (Figure 3.7). While on untreated control, dark soil, red soil and sandy loam soil (+), no accumulation of cucurbitacin A was detected (Figure 3.7). In contrast, the accumulation of cucurbitacin B residues on tomato plants treated by Nemarioc-AL phytonematicide was detected on dark, red, silt, sandy and sandy loam soil (+) with average mean of 0.023, 0.022, 0.023, 0.05 and 0.02 mg/kg, respectively. Nemafric-BL phytonematicide treated roots had accumulation of cucurbitacin B, silt soil attained higher accumulation of cucurbitacin B with an average mean of 0.05 mg/kg, followed by sandy soil and sandy loam soil (+) with an average of 0.04 mg/kg (Figure 3.8). The next one was red soil with 0.03 mg/kg, dark soil was the least on treated soil with 0.02 mg/kg (Figure 3.8). No accumulation of cucurbitacin A was detected in roots of Nemafric-BL phytonematicide treated soil (Figure 3.8).

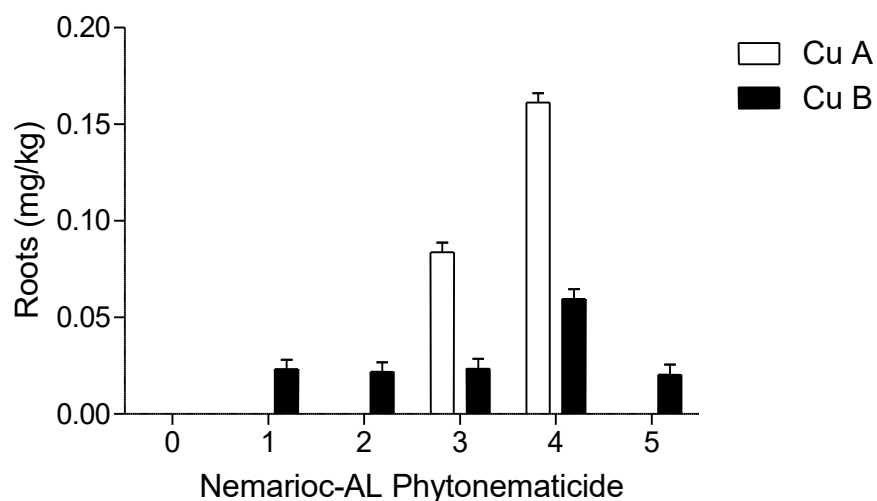


Fig 3.7. The effect of Nemarioc-AL phytonematicide on accumulation of cucurbitacin A and B residues in tomato roots under sandy loam, dark soil, red soil, sandy soil, and sandy loam (+) (n=36). Cu A = cucurbitacin A; Cu B = cucurbitacin B.

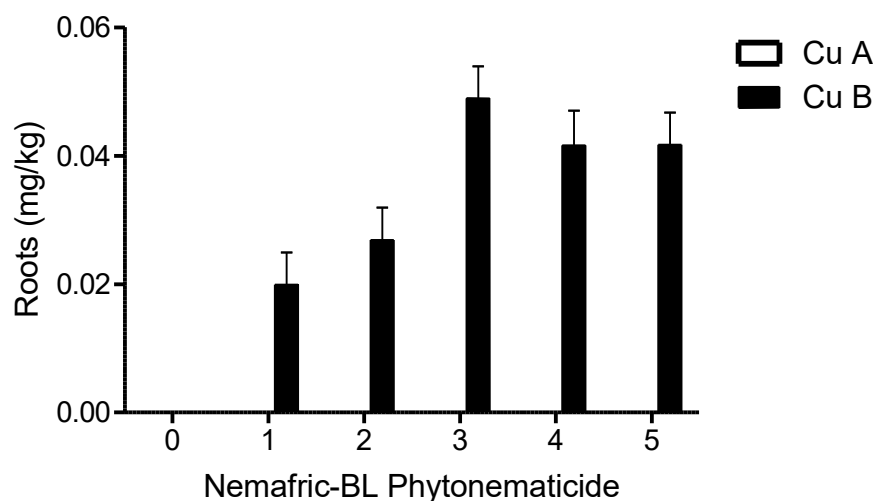


Fig 3.8. The effect of Nemarioc-AL phytonematicide on accumulation of cucurbitacin A and B residues in tomato roots under sandy loam, dark soil, red soil, sandy soil, and sandy loam (+) (n=36). Cu A = cucurbitacin A; Cu B = cucurbitacin B.

A high concentration of cucurbitacin A detected on Nemarioc-AL phytonematicide treated soil (silt, sandy soil) indicates abundance of cucurbitacin A in Nemarioc-AL phytonematicide. The accumulation of cucurbitacin A in Nemarioc-AL phytonematicide treated soil confirm that the fruits of *C. myriocarpus* possess a bioactive compound cucurbitacin A (Chen *et al.*, 2005), making it available in the product. In contrast, dark, red and sandy loam soil (+) exhibited no traces of cucurbitacin A in roots showing that cucurbitacin was translocated into plant parts. This could be that the cucurbitacin A is easily translocated into plant parts in the ascending sap with mineral and water. Because cucurbitacin A is partially non-polar and can slightly dissolve in water for plant uptake. Therefore, this study reveals that there is a small accumulation of cucurbitacin B in Nemarioc-AL phytonematicide. In contrast, Nemafric-BL phytonematicide treated roots exhibited only the accumulation of cucurbitacin B residues. The intensified accumulation of cucurbitacin B could be that *C. africanus* fruits contains cucurbitacin B which is non-polar and cannot be easily biodegraded.

3.3.5 Detecting cucurbitacin residues in soil

The accumulation of cucurbitacin A and B were not detected on sandy loam, dark soil, red soil, silt soil, sandy soil, and sandy loam (+) treated by Nemarioc-AL phytonematicide. The results corroborate with finding by Bango (2019) were the stated phytonematicide did not leave residues in soil. This could be that Nemarioc-AL as the biopesticide was easily biodegradable in the soil leaving no traces. Nemarioc-AL was made by effective microorganisms, molasses and fruits extracts from *C. myriocarpus* fruits (Mashela *et al.*, 2017). Just like other biopesticides, if they are applied, they easily become biodegradable leaving no traces as compared to many synthetic

chemical nematicides with persistent chemical residues (Soyel *et al.*, 2022). In contrast, there were traces of cucurbitacin B on dark, red and sandy loam soil (+) treated by Nemafric-BL phytonematicide with average mean of 0.03, 0.02 and 0.02, respectively (Figure 3.10.). While silt soil and sandy loam showed non-accumulation of cucurbitacin's. Leaving no traces of cucurbitacin A on all stated soil types. As shown, cucurbitacin B detected suggest that it biodegrades took place but slowly compared to cucurbitacin A. As depicted by traces of cucurbitacin B on dark, red and sandy loam soil (+), and non-traces of cucurbitacin B in silt and sandy soil.

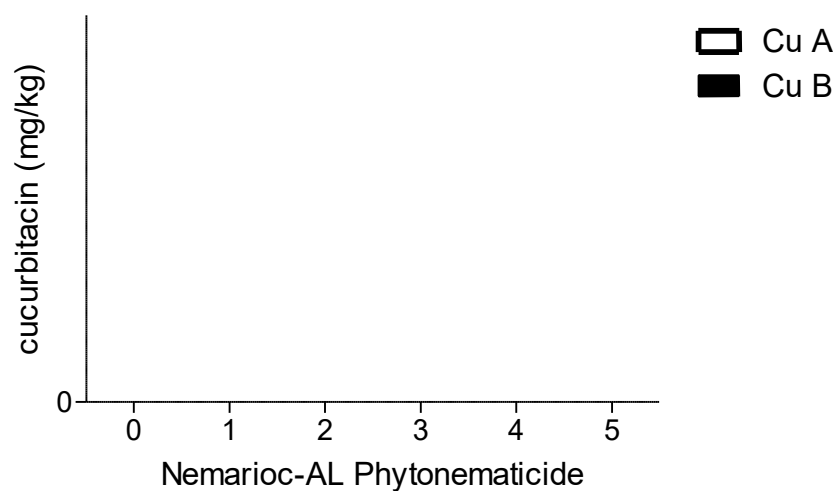


Fig 3.9. The effect of Nemarioc-AL phytonematicide on accumulation of cucurbitacin A and B residues in sandy loam, dark soil, red soil, sandy soil, and sandy loam (+) (n=36). Cu A = cucubitacin A; Cu B = cucubitacin B

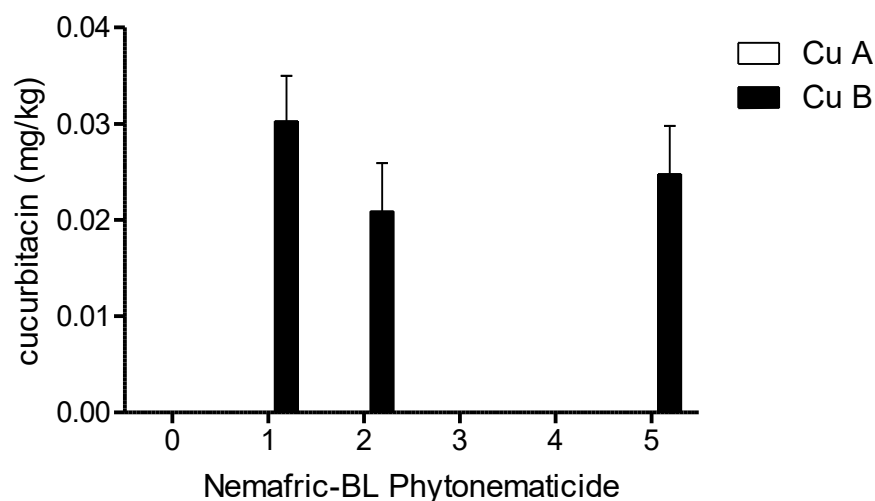


Fig 3.10. The effect of Nemarioc-AL phytonematicide on accumulation of cucurbitacin A and B residues in sandy loam, dark soil, red soil, sandy soil, and sandy loam (+) (n=36). Cu A = cucurbitacin A; Cu B = cucurbitacin B

Table. 3.1 Soil texture for sandy loam, dark soil, red soil, silt soil, sandy soil and Sandy loam (+) at planting.

Soil Types	Soil texture			OM (%)	Reference
	Sand (%)	Clay (%)	Silt (%)		
Sandy loam	65	30	5	1-6	Ramputla, 2019
Dark soil	22	40	38	5-12	Dube, 2016
Red soil	65	7	28	0.5-3	Jitendra Kumar et al., 2013
Silt soil	8	12	80	2-6	Okalebo et al., 2002.
Sandy soil	89	6	5	0.5-2	Dube, 2016
Sandy loam (+)	65	30	5	1-6	Ramputla, 2019

Table 3.2 The pH and EC for sandy loam, dark soil, red soil, silt soil, sandy soil and Sandy loam (+) at planting (IN) and after (FN) 150 days of application of Nemarioc-AL phytonematicide.

Soil types	pH (IN)	pH (FN)	EC dS/m (IN)	EC dS/m (FN)
Sandy loam	7,12	5,41	50,5	78,83
Dark soil	8,52	7,59	169,8	200,80
Red soil	7,93	5,82	77,3	128,20
Silt soil	7,66	5,32	32,8	76,13
Sandy soil	7,4	5,0	19,98	45,43
Sandy loam (+)	7,01	4,71	7,05	86,93

Table 3.3 The pH and EC for sandy loam, dark soil, red soil, silt soil, sandy soil and Sandy loam (+) at planting (IN) and after (FN) 150 days of application of Nemafric-BL phytonematicide.

Soil types	pH (IN)	pH (FN)	EC dS/m (IN)	EC dS/m (FN)
Sandy loam	7,06	5.55	54,50	47,90
Dark soil	8.51	7.26	225,00	207,60
Red soil	7.91	5.22	74,83	74,33
Silt soil	7.75	4.57	98,83	10,58
Sandy soil	7,37	4.65	69,47	85,70
Sandy loam (+)	7.09	4.83	89,20	7,08

There were no significant difference on the influence of organic matter content and soil texture on accumulations of cucurbitacin residues in the current study. The effects of Nemarioc-AL and Nemafric-BL phytonematicides on sandy loam soil, dark soil, red soil, silt soil, sandy soil and sandy loam soil (+) on pH and soil electrical conductivity have displayed either a decrease or an increase of the respective variables (Table 3.2 and 3.3). Nemarioc-AL and Nemafric-BL phytonematicides reduced pH levels of all the stated soil types as comparing a pH prior planting and after planting (Table 3.2 and 3.3). A reduction of pH in soil types could be equivalent to the actual pH of phytonematicides which is 3.7 (Mashela *et al.*, 2017). As a result, a long use of the phytonematicides will drastically reduce the soil pH to acidic as observed in the current

study. If a soil type becomes acidic, it will interrupt the normal chemical properties of the soil, resulting in the dissolution of some chemical compounds for plant uptake. This will increase the promote accumulation of residues in plant parts, which is a serious constraint for crop production (Hue, 2011). Acidic soil resulting from phytonematicides will negatively impact macro- and microorganisms and decrease the degradation of toxic substances, leading to prolonged bioavailability in the soil (Getaneh and Kidanemariam, 2021).

In the current study, Nemarioc-AL phytonematicide increased soil electrical conductivity on the stated soil types. These results corroborate with findings by Mashela, (2002), where Nemarioc-AG phytonematicide increased electrical conductivity level. An increase of electrical conductivity is generally undesirable as it depicts higher salinity and plant stress. Hence, increasing the chance for accumulation of residues in produce. In contradict with findings on tomato planted under hydroponic system, high concentrations of electrical conductivity increased growth and yield (Rosadi *et al.*, 2014). Similarly, Nemafric-BL phytonematicide in the current research reduced soil electrical conductivity. A decrease of soil electrical conductivity is usually ideal to avoid salinity. Soil electrical conductivity need to be maintained for each crop as it influences residue accumulations. Despite of trends observed on soil electrochemical conductivity before and after plant in the current study. The observed electrical conductivity was too high. This could be that the soil has already had accumulation of soluble salts like potassium, nitrogen and phosphate prior it was used. Additionally, all the soil types were collected from agricultural areas where synthetic chemical fertilisers are used on a daily basis.

3.4 Conclusion

Nemarioc-AL and Nemafric-BL phytonematicides reduced the pH levels of selected soil types. On the other side, Nemarioc-AL phytonematicide increased the soil electrical conductivity leading some soil types to acidic environment which could promote residues. The accumulation of both cucurbitacin A and B residues were detected in tomato plant parts with small quantities that will not induce toxicity on human beings. The given Nemarioc-AL and Nemafric-BL phytonematicides concentration 3% appeared to be safe and should be used for intended purposes in plant protection.

3.5 References

- Azlay, L., E I Boukhari, M. E. M., Mayad, E. H. and M. Barakate. 2023. Biological management of root-knot nematodes (*Meloidogyne* spp.): a review. *Organic Agriculture* 13: 99-117.
- Bango, H. 2019. Cucurbitacin chemical residues, non-phytotoxic concentration and essential mineral elements of Nemarioc-AL and Nemafric-BL phytonematicides on growth of tomato plant. Mini-dissertation, University of Limpopo, Sovenga, South Africa.
- Campbell, N.A. 1990. Biology. Benjamin/Cummings Publisher: Redwood City, United States of America.
- Chen, J.C., Chiu, M.H., Nie, R.L., Cordell, G.A. and S.X. Qiu. 2005. The cucurbitacins and cucurbitane glycosides: Structures and biological activities. *Natural Products Report* 22:386-399.

- Collins, E.J., Bowyer, C., Tsouza, A. and M. Chopra. 2022. Tomatoes: An extensive review of the associated health impacts of tomatoes and factors that can affect their cultivation. *Biology*, 11:1-45.
- Dube, Z.P. 2016. Nemarioc-AL and Nemafric-BL phytonematicides: Bioactivities in *Meloidogyne incognita*, tomato crop, soil type and organic matter. PhD Thesis, University of Limpopo, Sovenga, South Africa.
- Getaneh, S. and W. Kidanemariam. 2021. Soil acidity and its managements: a review. *International Journal of Advanced Research in Biological Sciences* 8:70-79.
- Grubben, G.J.H. and O.A. Denton. 2004. Plant Resources of Tropical Africa 2. Vegetables; PROTA Foundation: Wageningen, The Netherlands, pp. 261–268.
- Hodson, M.E., Vijver, M.G. and W.J. Peijnenburg. 2010. Bioavailability in soils. In *Dealing with contaminated sites: From theory towards practical application*. Dordrecht: Springer Netherlands.
- Hue, N.V. 2011. Alleviating soil acidity with crop residues. *Soil Science* 176: 543-549.
- J. R. Okalebo, K. W. Gathua and P. L. Woomer. 2002. Laboratory methods of soil and plant analysis. A Working Manual. Soil Science Society of East Africa Technical Publication no. 1, Nairobi, Kenya.
- Jitendra Kumar, J.K., Thomas, T. and A.K Shukla. 2013. Study of red soil and their physico-chemical properties around Bundelkhand Region in Uttar Pradesh.

- Kanissery, R., Gairhe, B., Kadyampakeni, D., Batuman, O. and Alferez, F., 2019. Glyphosate: Its environmental persistence and impact on crop health and nutrition. *Plants* 8:2-11.
- Kyan, T., Shintani, M., Kanda, S., Sakurai, M., Ohashi, H., Fujisawa, A. and S. Pongdit. 1999. Kyusei Nature Farming and the Technology of Effective Microorganisms. Asia Pacific Natural Agriculture Network: Bangkok, Thailand.
- Malebe, A.L. 2019. Potential cucurbitacin chemical residues and non-phytotoxic concentration of two phytonematicide formulations in nightshade
- Mashela, P.W. 2002. Ground wild cucumber fruits suppress numbers of *Meloidogyne incognita* on tomato in micro plots. *Nematropica* 32:13-19.
- Mashela, P.W., De Waele, D., Dube, Z., Khosa, M.C., Pofu, K.M., Tefu, G., Daneel, M.S. and H. Fourie. 2017. Alternative Nematode Management Strategies. In: Fourie, H., Spaull, V.W., Jones, R.K., Daneel, M.S. and D. De Waele (eds.). Nematology in South Africa: A View from the 21st Century. Springer International Publishers: Heidelberg.
- Mashela, P.W., Dube, Z.P. and K.M. Pofu. 2015. Managing the Phytotoxicity and Inconsistent Nematode Suppression in Soil Amended with Phytonematicides. In: Meghvansi, M.K. and A. Vorma (eds.). Organic Amendments and Soil Suppressiveness in Plant Disease Management, Soil Biology 46. Springer International Publishers: Heidelberg, Switzerland.
- Motulsky, H.J., 2007. Prism 5 statistics guide, 2007. GraphPad Software 31:39-42.
- Okalebo, J.; Gathua, K.; and P. Woomer. 2002. Laboratory Methods of Soil and Water Analysis: Nairobi, Kenya.

- Onkendi, E.M., Kariuki, G.M., Marais, M. and L.N. Moleleki. 2014. The threat of root-knot nematodes (*Meloidogyne* spp.) in Africa: A Review. *Plant Pathology* 63:727-737.
- Ramputla, M.J. 2019. Nutritional water productivity of hot chilli (*Capsicum annuum*) under infection with *Meloidogyne javanica* and *Meloidogyne incognita* race 2. Mini-dissertation, University of Limpopo, Sovenga, South Africa.
- Rosadi, R.B., Senge, M., Suhandy, D. and Tusi, A., 2014. The effect of EC levels of nutrient solution on the growth, yield, and quality of tomatoes (*Solanum lycopersicum*) under the hydroponic system. *Journal of Agricultural Engineering and Biotechnology* 2: 1-12.
- Schaal, B., 2019. Plants and people: Our shared history and future. *Plants, People, Planet* 1:14-19.
- Seshweni, M.D. 2016. Integrated system for the management of *Meloidogyne javanica* in potato production. Mini-dissertation, University of Limpopo, Sovenga, South Africa.
- Shadung, K.G. 2016. Quality protocols for Nemarioc-AL and Nemafric-BL phytonematicides and potential chemical residues in tomato fruits. PhD Thesis, University of Limpopo, Sovenga, South Africa.
- Shaik, R.S., Burrows, G.E., Urwin, N.A., Gopurenko, D., Lepschi, B.J. and L. Weston, 2017. The biology and management of prickly paddy melon (*Cucumis myriocarpus* L.), an important summer annual weed in Australia. *Crop Protection*, 92:29-40.
- Soyel, S.A., Hazra, D.K., Ruidas, S., Mandal, S., Bhattacharyya, S., Poi, R., Karmakar, R., Mondal, G., Majumder, S. and P. Mondal. 2023. Simultaneous monitoring and decontamination of pesticide residues in phytomedicine-

- enriched betel leaf utilizing QuEChERS-GC-MS/MS technology to safeguard public health. *Journal of AOAC International*, 106:1209-1219.
- Takla, S.S., El-Dars, F. M., Amien, A.S. and M. A. Rizk. 2021. Prospects of Neem essential oil as bio-pesticide and determination of its residues in eggplant plants during crop production cycle. *Egyptian Academic Journal of Biological Sciences, F. Toxicology and Pest Control*, 13: 1-15.
- Traoré, A., Bandaogo, A. A., Savadogo, O. M., Saba, F., Ouédraogo, A.L., Sako, Y., Sermé, I. and S. Ouédraogo. 2022. Optimizing Tomato (*Solanum lycopersicum* L.) Growth with Different Combinations of Organo-Mineral Fertilizers. *Frontiers in Sustainable Food Systems*, 5: 1-7.
- Yu, J., Pressoir, G., Briggs, W.H., Vroh Bi, I., Yamasaki, M., Doebley, J.F., McMullen, M.D., Gaut, B.S., Nielsen, D.M., Holland, J.B. and S. Kresovich. 2006. A unified mixed-model method for association mapping that accounts for multiple levels of relatedness. *Nature genetics* 38:203-208.

CHAPTER 4

THE EFFECTS OF NEMARIOC-AL AND NEMAFRIC-BL PHYTONEMATICIDES ON TOMATO PLANTS INOCULATED BY *ESEZIA FETIDA* UNDER GREENHOUSE ENVIRONMENT

Abstract

Earthworms play an important role in tomato production by decomposing organic matter and improving the physical characteristics of the soil. They are regarded as an indicator of soil pollution due to their high sensitivity to toxic substances. They coexist with plant-parasitic nematodes in the soil. During nematode management, earthworms are also exposed to Nemarioc-AL and Nemafric-BL phytonematicides. Therefore, it's crucial to develop non-toxic concentrations of phytonematicides in earthworms and tomato plants. The objective of this study was to assess the toxicity of Nemarioc-AL and Nemafric-BL phytonematicides on earthworms and protein synthesis. Separate trials of Nemarioc-AL or Nemafric-BL phytonematicide were conducted concurrently under a greenhouse environment. Treatment constituted 0, 2, 4, 8, 16, 32 and 64% Nemarioc-AL or Nemafric-BL phytonematicide arranged in RCBD. At harvest, plants, earthworms, and proteomics were analyzed. The MCSP Nemarioc-AL and Nemafric-BL phytonematicides developed on tomato plants inoculated with earthworms were 1.09 and 1.35%, respectively. Earthworms' variables, number of earthworms (NW), number of eggs (NE), final mass (FM) and final population (Pf) to Nemarioc-AL and Nemafric-BL phytonematicides exhibited either positive or negative quadratic relations. Therefore, the developed concentrations will assist farmers to avoid toxicity on non-target organisms.

Keywords: earthworm, organic matter, inoculated, nematodes, toxicity

4.1 Introduction

Nemarioc-AL and Nemafric-BL phytonematicides are newly developed biopesticide products to manage plant parasitic nematodes on various crops. The efficacy of the products on plant parasitic nematode suppression depends on the active ingredients cucurbitacin A ($C_{32}H_{46}O_9$) and cucurbitacin B ($C_{32}H_{46}O_8$), respectively, which are toxic to the stated species (Mashela *et al.*, 2024). At higher concentrations, phytonematicides also induced toxicity in tomato crops (Bango *et al.*, 2019); Swiss chard (Mashela *et al.*, 2021) and *Pelargonium sidoides* (Sithole *et al.*, 2016). However, since plant parasitic nematodes interact with other beneficial organisms in the soil ecosystem, there is a need to assess how phytonematicides affect earthworms using proteomics. An earthworm is a soil-dwelling terrestrial invertebrate that belongs to the phylum Annelida (Kamdem *et al.*, 2019). They are divided into three categories namely epigeic, endogeic and anecic (De Wandeler *et al.*, 2016). Due to their burrowing behaviour and the nutrients, they provide to the soil through their waste products, earthworms are extremely essential soil invertebrates. They improve the physical characteristics of the soil, the cycling of nutrients through the soil, and the activity of other beneficial soil organisms. Given their high sensitivity to anthropogenic contaminants, earthworms have been employed as indicator animals in soil pollution assessments.

Globally, approximately 2 million tonnes of synthetic chemical pesticides, which cause a fortune are used annually in plant protection to prevent crop loss due to pests and diseases (Devi *et al.*, 2017). However, these synthetic chemical pesticides have a negative impact on the environment, soil organisms, animals and human beings (Yatoo *et al.*, 2022). Earthworms are negatively impacted by such chemical intrusion

in soil at various phases of their life cycle (Yatoo *et al.*, 2022). These effects include mortality, behavioural changes, decreased growth, reproduction, and other biochemical effects. Coiling, curling, extrusion of coelomic fluid, segmental constriction, and swelling were among the typical symptoms (Yuguda *et al.*, 2015). The detrimental impacts of these synthetic chemical pesticides on soil ecosystems—which are employed to protect plants—are well documented, but there is still uncertainty regarding their toxicity and proteomics-related risks (Zhao *et al.*, 2019). Proteomics is an analytical method that uses a variety of analytical instruments and procedures to identify and quantify the total amount of protein in a cell, tissue, or organism (Thikekar *et al.*, 2023). We can identify effects of Nemarioc-AL and Nemafric-BL phytonematicides on earthworms at proteome level. The objective of this study was to assess toxicity of Nemarioc-AL and Nemafric-BL phytonematicides on earthworms and proteins synthesis.

4.2 Materials and Methods

4.2.1 Description of study site

The study was conducted at Green Biotechnologies Research Center of Excellence, University of Limpopo, South Africa (23°53'10'S, 29°44'15'E). Separate trials for Nemarioc-AL and Nemafric-BL phytonematicides were conducted concomitantly in the greenhouse during autumn (March 2023) and validated in autumn (March 2024).



Figure 4.1. Tomato plants treated by Nemarioc-AL (A) and Nemafric-BL (B) phytonematicides under greenhouse conditions.

4.2.2 Treatments and Experimental Design

The treatments consisted of 0, 2, 4, 8, 32, 16, and 64% Nemarioc-AL and Nemafric-BL phytonematicides both arranged in randomized complete block design, replicated 6 times. Due to obvious sources of variations created by fans cure was taken when choosing this design.

4.2.3 Procedures and Cultural Practices

A locally created technique was used to prepare the Nemarioc-AL and Nemafric-BL phytonematicides (Mashela *et al.*, 2017). Tomato seeds were procured from vkb Polokwane and stored until required. The seeds were directly sown into 200-hole seedling trays that were filled with Hygromix-T (Hygrotech, Pretoria North) and allowed to grow in the greenhouse. At four leave stage, tomato seedlings were hardened off outside the greenhouse by withholding water and irrigating them when 80% are wilting for a period of 5 days prior planting. Greenhouse benches were set with 20 cm diameter plastic pots arranged at intra- and inter- row spacing of 0.3 m. Uniform tomato

plants were transplanted directly into 20-cm diameter pots filled with 2700 mL compost procured from Martindale nursery (Tzaneen). During the growing season, seedlings were fertilized with sea weed organic fertilizer and irrigation was accomplished with 100 mL of chlorine-free water. Seven days after transplanting, seedlings were inoculated with 12 earthworms per pod. Treatments were applied at seven days after inoculation and thereafter once on weekly basis. Scouting and monitoring for insect pests and diseases inside greenhouse were carried out on daily basis. Red spider mites (*Tetranychus urticae*), white fly (*Aleurodicus dispersus*) and leaf minor (*Tuta absoluta*) inside greenhouse were managed by using repellent plants basil (*Ocimum basilicum*) and insect traps.

4. 2.4 Proteomics

4.2.4.1 Test animals and chemicals

The earthworms red Wigglers (*Esenia fetida*) were collected from earthworm breeder Bio cast vermiculture South Africa Pretoria and ZZ2 Moeketsi Polokwane. The earthworms *Esenia fetida*, weighing between 300 and 600 mg, were purchased. After transported to a greenhouse without any physical and chemical stress, the worms were bred at ambient temperature of 20 °C inside a green house, using the dark soil mixed with kitchen waste. Every week, earthworms were fed by kitchen waste and soil was irrigated every three days to maintain the soil's humidity at approximately 70%. All trials were conducted using mature earthworms that weighed between 300 and 600 mg and had a fully developed clitellum.

4.2.4.1 Protein extraction

Proteins from earthworms were extracted according to the trichloroacetic acid-acetone (TCA-A) method through some modification (Wang *et al.*, 2010). In short, earthworms were collected from the pods and sorted according to treatments. Then 2 g from each sample was pulverized to powder by liquid nitrogen using a pestle and mortar. The protein was precipitated overnight using 8–10 times the amount of ice-cold TCA-A solution containing 20% TCA (w/v) and 0.1% DTT (w/v) in a 50 mL centrifuge tube holding the powdered homogenate. The precipitate was centrifuged for 40 minutes at 4 °C and 12000 × g. It was then rinsed twice with 30 mL of pre-chilled acetone solution that contained 0.1% DTT (w/v) and once with the 80% washing solution above. The resultant pellets were redissolved in lysis buffer [7 M urea, 2 M thiourea, 4% CHAPS (w/v), 65 mM DTT, and 0.5% (w/v) Bio-lyte (pH 4–6 and pH 5–7, Bio-rad, USA)] after being vacuum freeze dried until all of the remaining acetone had been removed. The ultrasonic cell disruptor (JY92-II Ultrasonic Crasher, Ningbo Scientz Biotechnology Co., Ltd, China) was used to sonicate the undissolved protein composition in the lysis buffer at 4 °C and 300 W for 40 apm (actions per minute). The mixture was then centrifuged at 4 °C and 13500 × g for 40 minutes. After collecting the supernatant, the Bradford's method was used to determine the protein content using Coomassie Brilliant Blue G-250 (Bio-Rad, USA) and BSA as a standard.

4.2.5 Data analysis

To generate biological indices for developing MCSP (Dm, Rh), curves, quadratic equations and the related biological indices (k), data for plant variables were subjected to CARD model (Liu *et al.*, 2003).

$$\text{MCSP} = D_m + (R_h/2)$$

Before the CARD model, the geometric series of the phytonematicide concentration was expressed as an exponential series ($2^0, 2^1, 2^2, 2^3, 2^4, 2^5$ and 2^6) and since $(\log_2 2) = x$, the log-transformed series was equivalent to 0, 1, 2, 3, 4, 5 and 6% (Tseke and Mashela, 2018). This was done to prevent overcrowded observations at lower concentration and to ensure the correct equidistance's between them. Plant variables that showed positive quadratic relations were used to generate the MCSP values for Nemarioc-AL and Nemafric-BL phytonematicides, with $R^2 \geq 0.25$ (i.e. $r = 0.5$). Earthworms were subjected to the line of best fit (Gomez and Gomez, 1984).

4.3 Results and Discussion

The data from the two seasons (experiment 1 and 2) were pooled ($n = 84$) and reanalysed since seasonal interactions were not significant for all variables (Gomez and Gomez, 1984).

4.3.1 Plant variables

The Curve-fitting Allelochemical Response Dose (CARD) algorithm model generated biological indices (Table 4.1) and related curve responses were depicted by figures. Response of dry shoot mass (DSM) (Figure 4.2), dry root mass (DRM) (Figure 4.3), plant height (PH) (Figure 4.4), stem diameter (SD) (Figure 4.5), number of flowers (NF) (4.6) to increasing concentrations of Nemarioc-AL phytonematicide exhibited positive quadratic relations with a model for respective variables constituting 97, 95, 98, 96 and 96% coefficients of determination. Chlorophyll content was not significantly affected by Nemarioc-AL phytonematicide. The response of plant variables to

increasing concentration of Nemarioc-AL phytonematicide has followed a density-dependent growth (DDG) pattern (Mashela *et al.*, 2017).

Plant variables and increasing concentrations of phytonematicide exhibited positive quadratic relations (Liu *et al.*, 2003), which was further conceptualised as a density-dependent growth (DDG) pattern (Mashela *et al.*, 2017). The DDG generated through CARD algorithm model was compartmentalized into three phases namely stimulation, neutral and inhibition phase (Mashela *et al.*, 2017). At lower concentration levels the phytonematicide stimulated growth of DSM, DRM, PH, SD, and NF. Suggesting that at lower concentrations they were no toxicity induced by the product on respective variables (Lebea, 2017; Malebe, 2019). As the concentrations of the phytonematicide increase, it reaches a point where stimulation stops technically known as neutral phase. At neutral phase neither stimulation or inhibition of DSM, DRM, PH, SD, and NF occurred at this stage. At this stage there were still no signs of toxicity observed on plant variables. The end of neutral phase, starts an inhibition phase, which occurs at higher concentration level. Inhibition phase was observed by a declining line demonstrating that plant variables were drastically reduced showing a sign of toxicity induced by the product. Toxicity was observed when the phytonematicide was used as post emergence bio-nematicide, then product inhibited seed germination indices (Mafeo and Mashela, 2010; Mafeo *et al.*, 2011b). On tomato plants treaded by

Nemarioc-AL phytonematicide at higher concentration levels, plant variables were also reduced (Bango, 2019; Sithole *et al.*, 2016).

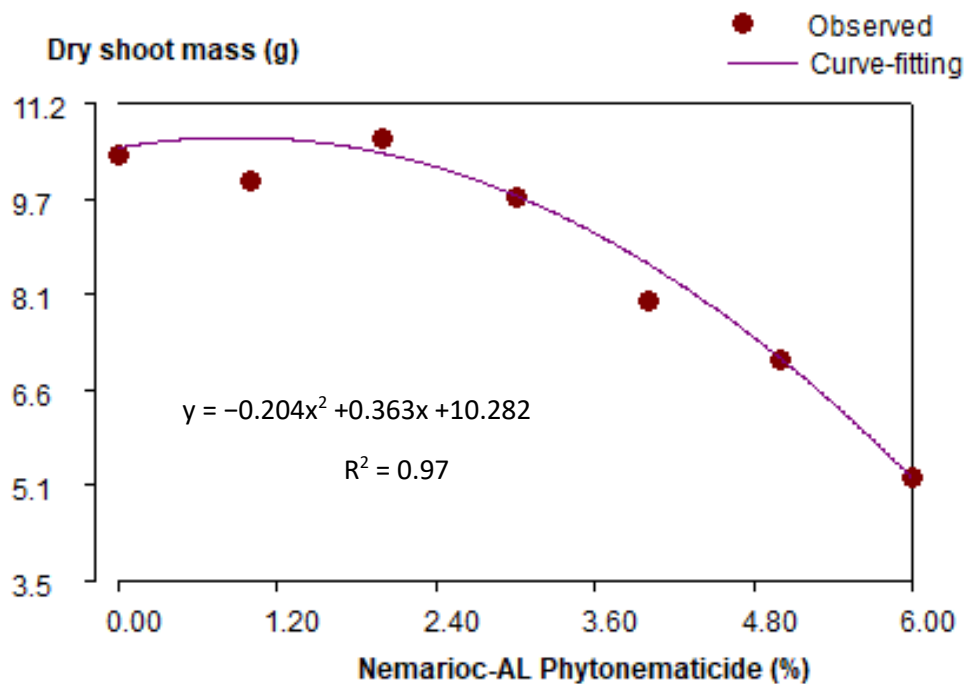


Fig 4.2 The effects of Nemarioc-AL and Nemafric-BL phytonematicides on dry shoot mass (DSM) at 56 days after application of treatment (n=72).

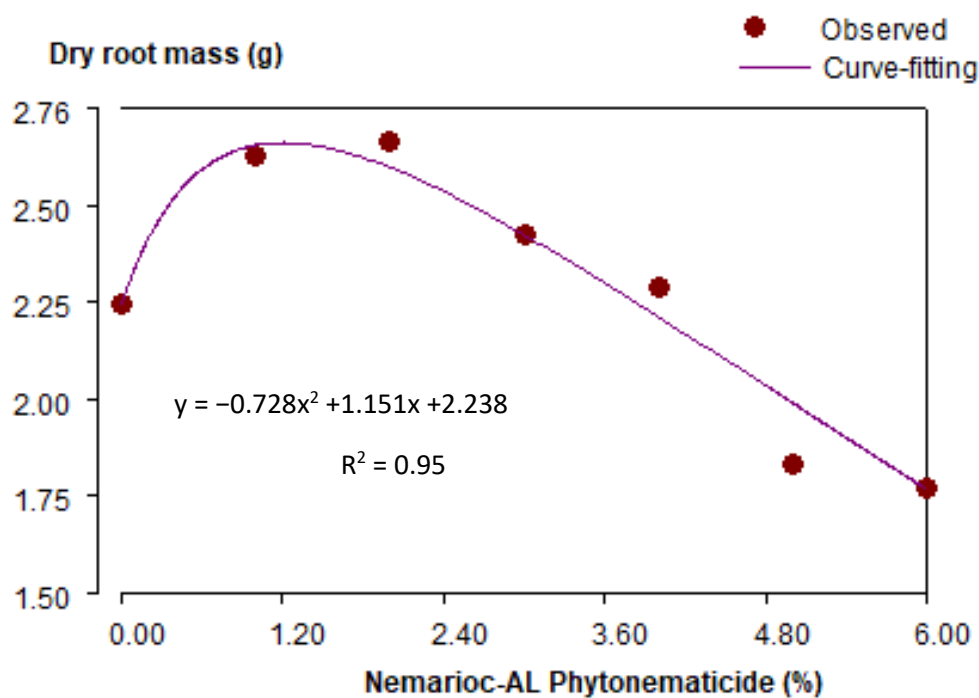


Fig 4.3 The effects of Nemarioc-AL phytonematicide on dry root mass (DRM) at 56 days after application of treatment (n=72).

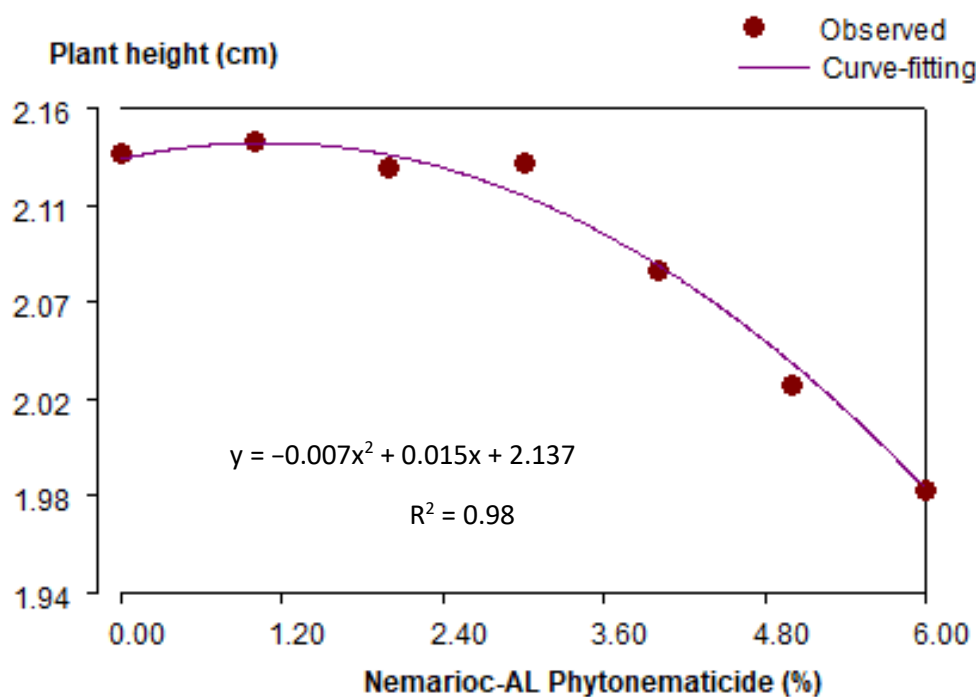


Fig 4.4 The effects of Nemarioc-AL phytonematicide on dry root mass (PH) at 56 days after application of treatment (n=72).

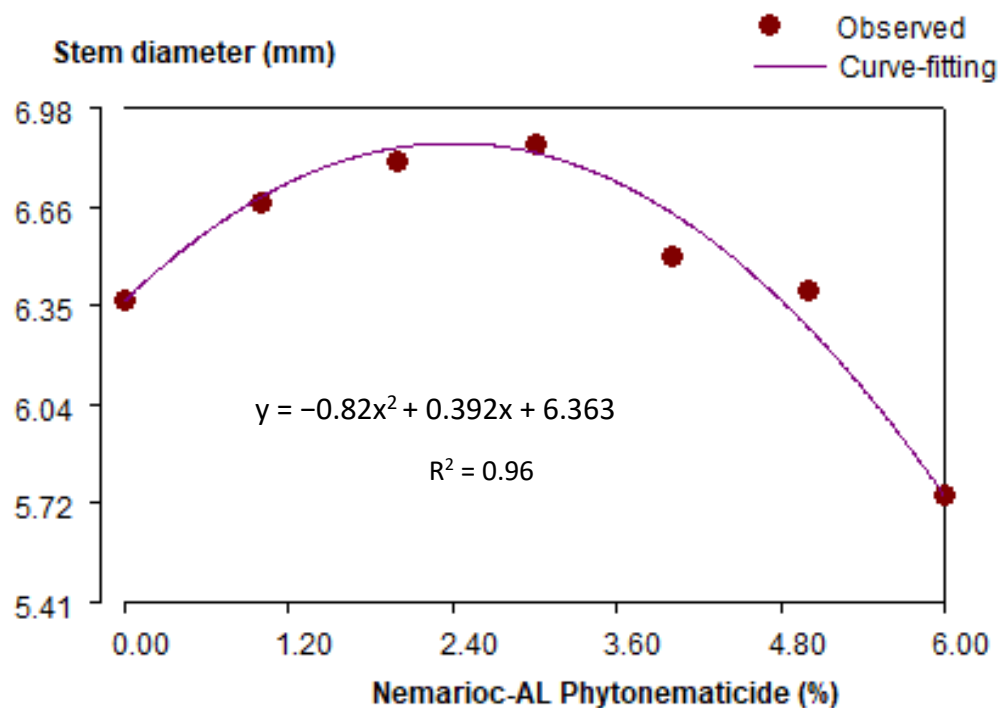


Fig 4.5 The effects of Nemarioc-AL phytonematicide on stem diameter (SD) at 56 days after application of treatment (n=72).

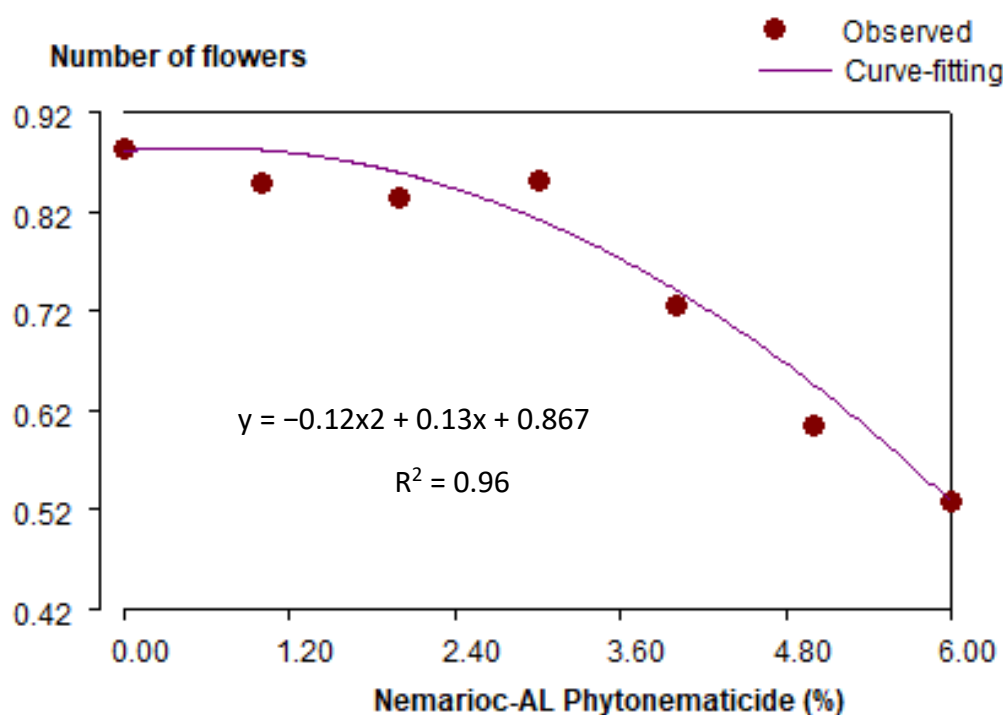


Fig 4.6 The effects of Nemarioc-AL phytonematicide on number of flowers (NF) at 56 days after application of treatment (n=72).

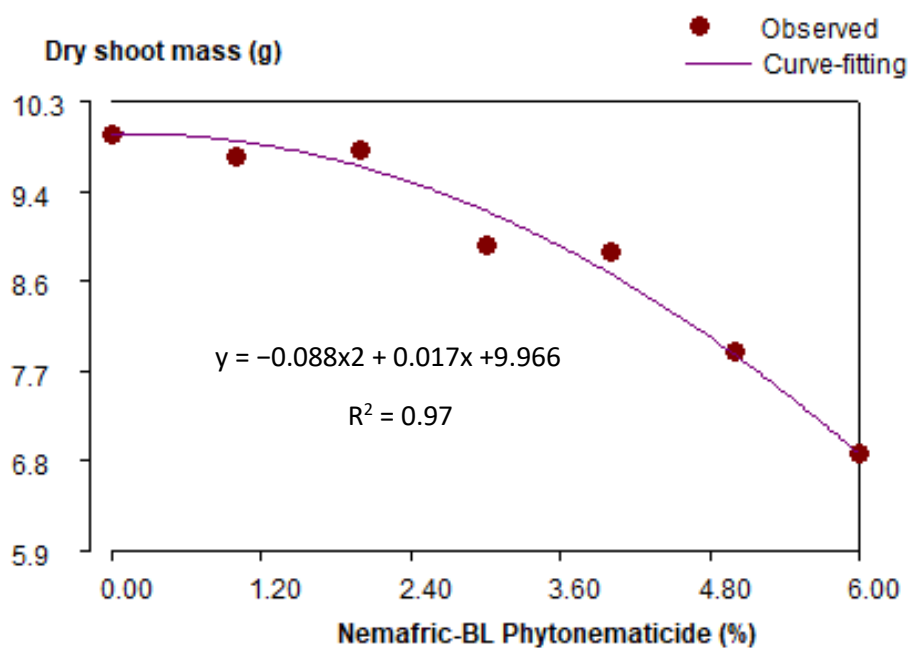


Fig 4.7. The effects of Nemafric-BL phytonematicide on dry shoot mass (DSM) at 56 days after application of treatment (n=72).

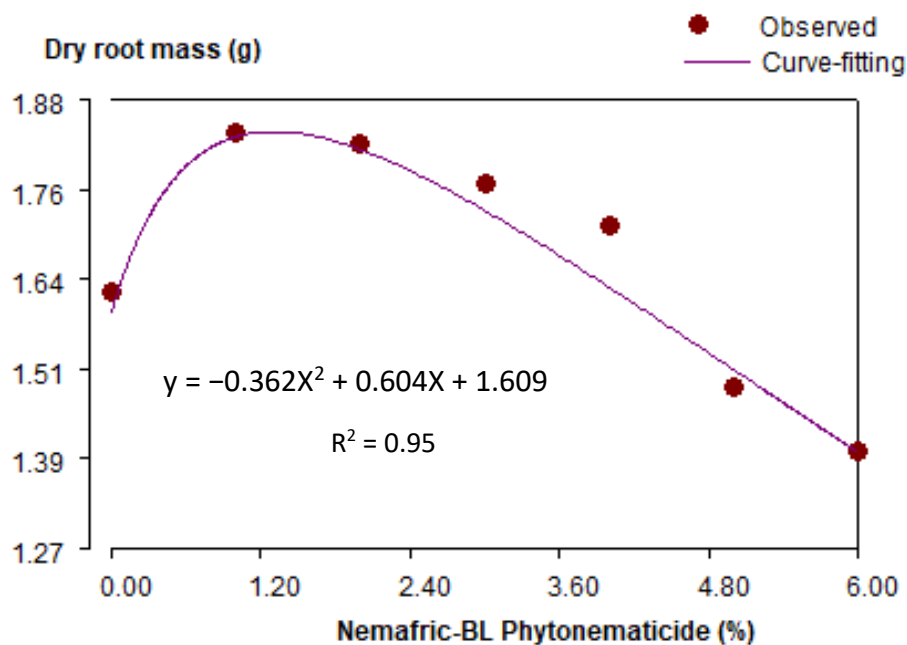


Fig 4.8 The effects of Nemafric-BL phytonematicide on dry root mass (DRM) at 56 days after application of treatment (n=72).

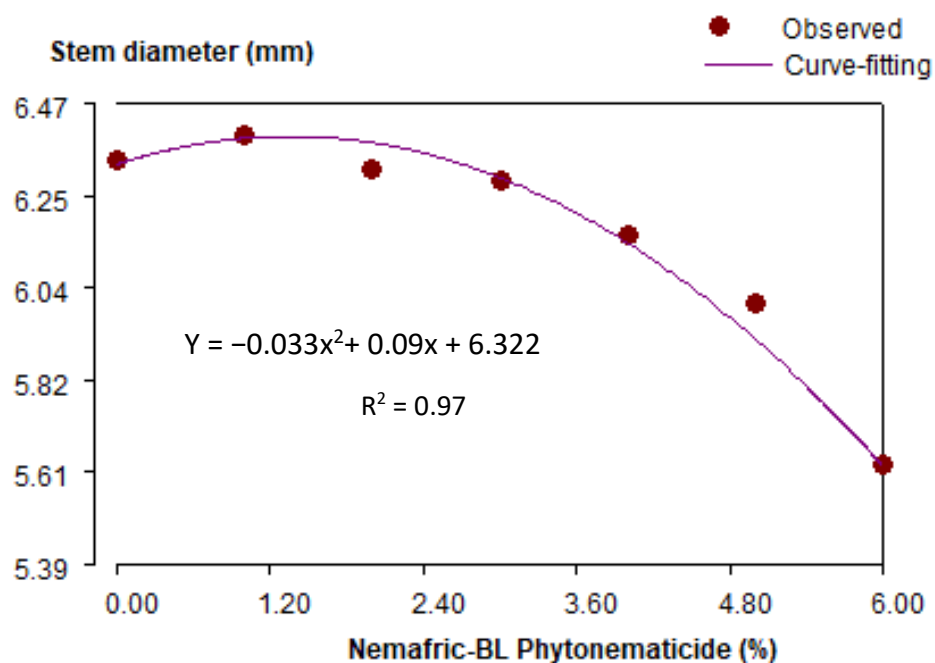


Fig 4.9 The effects of Nemafric-BL phytonematicide on plant height (PH) at 56 days after application of treatment (n=72).

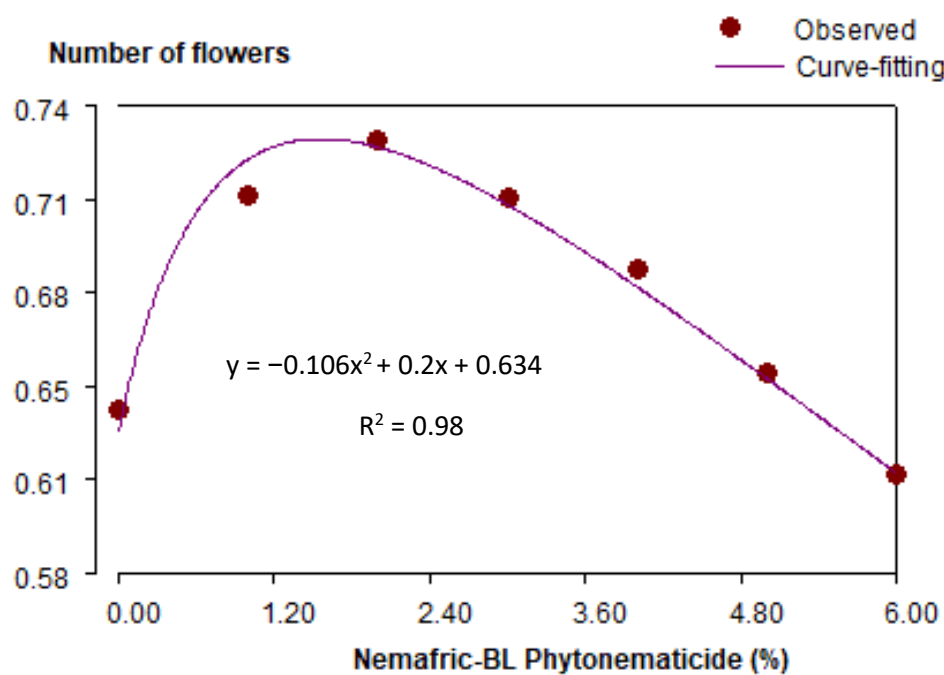


Fig 4.10 The effects of Nemafric-BL phytonematicide on stem diameter (SD) at 56 days after application of treatment (n=72).

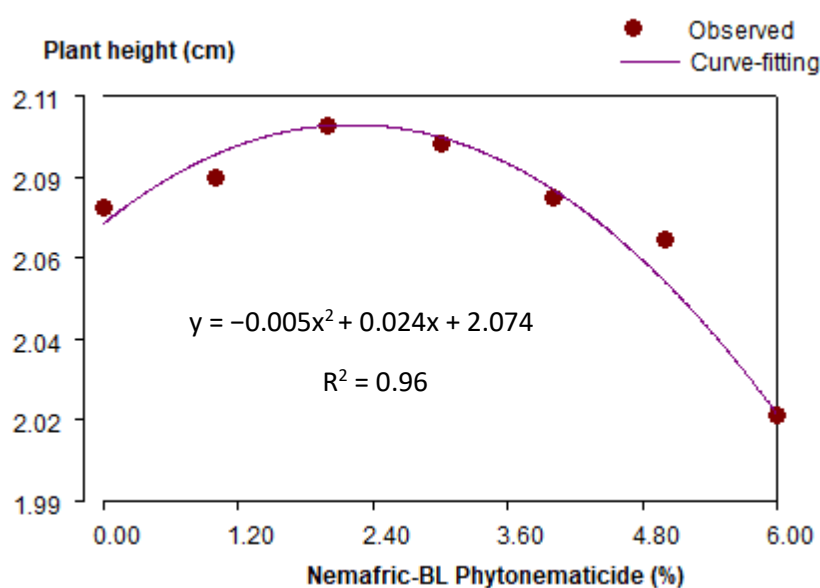


Fig 4.11 The effects of Nemafric-BL phytonematicide on number of flowers (NF) at 56 days after application of treatment (n=72).

Dry shoot mass (Figure 4.7), DRM (Figure 4.8), PH (Figure 4.9), SD (Figure 4.10) and NF (Figure 4.11) to increasing Nemafric-BL phytonematicide exhibited a positive quadratic equation with a model presented 97, 95, 96, 97 and 98%, coefficients of determination association, respectively. Chlorophyll content was not significantly affected by either Nemarioc-AL or Nemafric-BL phytonematicides. In all plant variables treated by Nemafric-BL phytonematicide they were strong allelopathic interaction between plant variables and the product as depicted by high coefficients of determination. Phytonematicide contain bioactive compound cucurbitacin B with allelopathic effects to other species (Mafeo and Mashela, 2010; Mafeo *et al.*, 2011b).

At low concentration level, Nemafric-BL phytonematicide had stimulation effects on DSM, DRM, PH, SD, and NF. Similar results were observed on tomato plants treated by Nemafric-BL phytonematicide where growth was stimulated at lower concentrations of phytonematicide (Mashela and Tseke, 2018; Pelinganga, 2013). While at higher

concentration levels, plant growth was inhibited. Generally, allelopathy is a phenomenon whereby secondary metabolites produced by living organisms influence the growth and development of other species either positively or negatively (toxicity) (Sithole, 2021). In the current study, as shown by CARD algorithm model generated biological indices the allelopathy had a positive effects of plant variables as shown by stimulation phase at lower concentrations of a product. However, at higher concentration level, allelopathy reduced plant variables as demonstrated by CARD algorithm model as indicated by inhibition phase. In such, negative effects of allopathy can be avoided by using lower concentration levels of Nemafric-BL phytonematicide.

Biological indices which exhibited positive quadratic relations, were used to compute the average MCSP values, since the former had a stimulation phase. The MCSP of Nemarioc-AL phytonematicide on tomato plants was successfully developed at 1.09%. The sensitivity (k) value for respective plant variables DSM, DRM, PH, SD and NF were 0,1, 0, 0 and 0, resulting in the overall sensitivity (Σk) value of 1 unit. The developed MCSP of 1.09 % on tomato plants treated by Nemarioc-AL phytonematicide was lower compared to the one developed on Swiss chard (*Beta vulgaris* L. cicla) of 3.03% (Mashela *et al.*, 2021). It was also low when compared to the developed MCSP previously developed on tomato plants treated by Nemarioc-AL phytonematicide of 2,64% (Pelinganga, 2013) and 4% (Tseke, 2013). The developed MCSP was close to the one developed on the same crop of 1.13% (Bango, 2016). The MCSP of citrus rootstock (*Volkameriana citrus* Pasq.) was high 8.6% compared to the one developed on tomato plants. If the MCSP developed was higher, its adjusted to 2% in such that

it will not induce toxicity to crops. The developed MCSP of 1.09% falls within the recommended one of 1.00% to 3.00% (Mashela *et al.*, 2021).

Using biological indices the overall sensitivity (Σk) of Nemarioc-AL phytonematicide on tomato had been 1 unit (Table 4.1). The Σk sensitivity of 1 indicates that this crop is sensitive to Nemarioc-AL phytonematicide. As indicated by (Liu *et al.*, 2003), the Σk sensitivity value that are close to zero, the more sensitive is a plant to a tested product. In contrast, sensitivity of a plant away from zero, is the more tolerant of a plant to a tested product. The overall sensitivity value previously generated on tomato plants treated by Nemarioc-AL phytonematicide was 3% (Pelinganga, 2013) and 60% (Bango, 2019). This indicates that this plant is more tolerant to Nemarioc-AL phytonematicide.

Table 4.1 Biological indices for dry shoot mass (DSM), dry root mass (DRM), plant height (PH), stem diameter (SD) and number of flowers (NF) of tomato plants to increasing concentration of Nemarioc-AL phytonematicide under greenhouse conditions.

	DSM	DRM	PH	SD	NF	Average
Biological index	Nemaroc-AL phytonematicide					
Threshold stimulation (D_m)	0.891	1.204	1.072	2.403	0.546	0.982
Saturation point (R_h)	0.162	0.455	0.008	0.471	0.004	0.22
0% inhibition (D_0)	1.782	3.858	2.144	4.806	1.093	2.736
50% inhibition (D_{50})	5.992	8.588	13.66	9.094	6.602	8.787
100% inhibition (D_{100})	8.1	14.1	18.8	11.6	9.1	12.34
R2	97	0.95	0.98	0.96	96	
Sensitivity (k)	0	1	0	0	0	
Overall sensitivity ($\sum k$) = 1						
Nemarioc-AL phytonematicide: MCSP = $D_m + (R_h/2) = 1.09\%$						

A Curve-fitting Allelochemical Response Dose (CARD) algorithm model computed the MCSP through generated biological with positive quadratic relations. The MCSP established on tomato plants inoculated with earthworms treated by Nemafric-BL phytonematicide was 1.35%. The established MCSP 1.35% in the current study fall within the recommended range for 1.00 to 3.00 previously established on tomato plants 2.64% (Pelinganga, 2013) and 1.75% (Bango, 2019). In some crops, the MCSP 5.31% was higher as observed on 3.08% Nightshade (*Solanum retroflexum* Dun.) (Malebe, 2019). On other phytonematicide developed from Tickberry (*Lantana camara* L.) technically known as Nemalan, MCSP developed was 5.31% (Malatjie, 2017). The higher MCSP values were further adjusted to 2%, based on MCSP is a concentration that will not induce toxicity. The sensitivity (k) value for respective plant variables DSM,

DRM, PH, SD and NF were 0,1, 0, 0 and 1, resulting in the overall sensitivity (Σk) value of 2 units. The Σk computed on tomato plants indicates plant tolerant to a product. In contrast, the sensitivity observed from nightshade was 0 when a Nemafric-BL phytonematicide was applied (Malebe, 2019). Indicates that this plant was very sensitive to a product. Additionally, overall sensitivity of tomato plants to Nemafric-BL was also shown to be 0 (f, 2017). Showing that some biopesticides are sensitive compared to Nemafric-BL phytonematicide.

Table 4.2 Biological indices for dry shoot mass (DSM), dry root mass (DRM), plant height (PH), stem diameter (SD) and number of flowers (NF) of tomato plants to increasing concentration of Nemafric-BL phytonematicide under greenhouse conditions.

	DSM	DRM	PH	SD	NF	Average
Biological index	Nemafric-BL phytonematicide					
Threshold stimulation (D_m)	0.096	1.304	2.211	1.351	1.561	0.982
Saturation point (R_h)	0.001	0.252	0.027	0.061	0.094	0.22
0% inhibition (D_0)	0.192	4.308	4.422	2.703	5.556	2.736
50% inhibition (D_{50})	7.623	11.714	16.188	11.19	17.288	8.787
100% inhibition (D_{100})	10.7	21.2	21.9	15.2	34.1	12.34
R ²	97	95	96	97	98	
Sensitivity (k)	0	1	0	0	1	
Overall sensitivity (Σk) = 2						
Nemafric-BL phytonematicide: MCSP = $D_m + (R_h/2) = 1.35\%$						

4.3.2. Earthworm variables

The response of number of earthworms (NW), number of eggs (NE), final mass of earthworms (ME) and final population (Pf) to increasing concentrations of Nemarioc-AL phytonematicide exhibited positive quadratic relations with model of the respective variables explained 0.95, 0.76, 0.95 and 0.92% coefficient of determination, respectively. The stated phytonematicide stimulated NW, NE, ME and Pf showing no signs of toxicity on the at lower and higher concentration levels. This confirms with findings by Mashela *et al.* (2024), which states that the cucurbitacin-containing phytonematicides did not affect non-target organisms like insects and bacteria. This was confirmed by application of cucurbitacin-containing phytonematicide on entomopathogenic nematode, *Steinernema feltiae* (Madaure *et al.*, 2017). *Steinernema feltiae* have shown tolerance to cucurbitacin-containing phytonematicide as compared to plant parasitic nematodes. The current results confirm with findings by Tseke and Mashela (2017), where Nemarioc-AL phytonematicide decreased nematode population numbers on tomato plants. Mashela *et al.*, (2021), also reported a decrease in nematode population densities on Swiss chard inoculated by *Meloidogyne enterolobii*. A decrease of plant parasitic nematodes number is economic important, as nematodes cause a drastic yield loss of over 50% to complete crop failure (Sithole, 2016). An increase of earthworms on tomato plants treated by Nemarioc-AL phytonematicide was beneficial in the current study. Because earthworms improve soil physical characteristics, by recycling of nutrients through the soil, and the activity of other beneficial soil organisms.

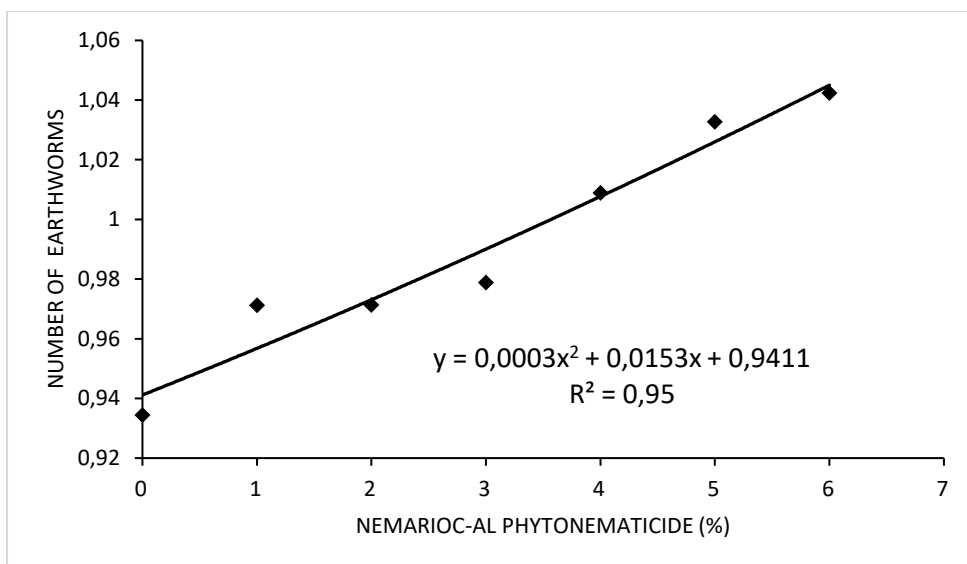


Fig 4.12 Response of number of earthworms (NW) to increasing concentrations of Nemarioc-AL phytonematicides (n=72).

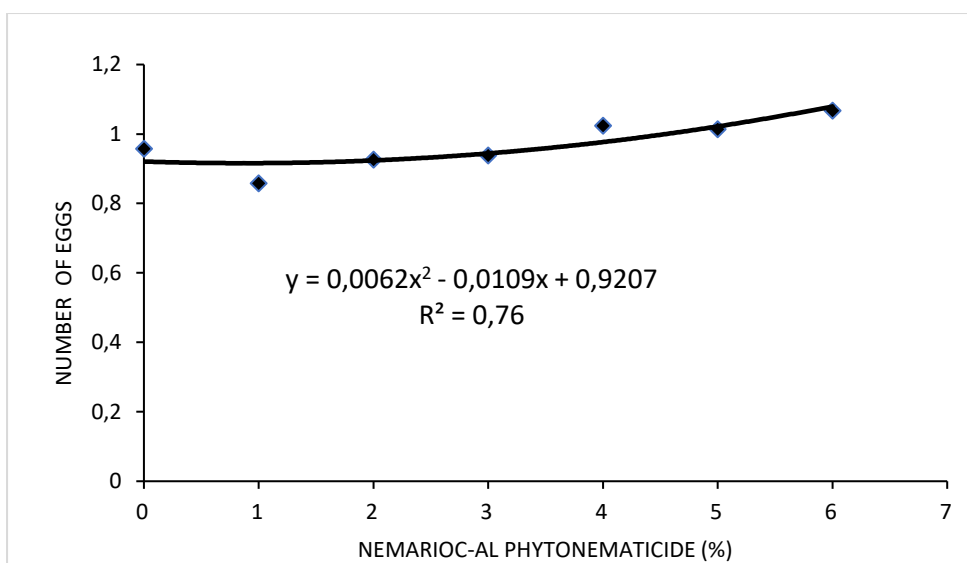


Fig 4.13 Response of number of eggs (NE) to increasing concentrations of Nemarioc-AL phytonematicides (n=72).

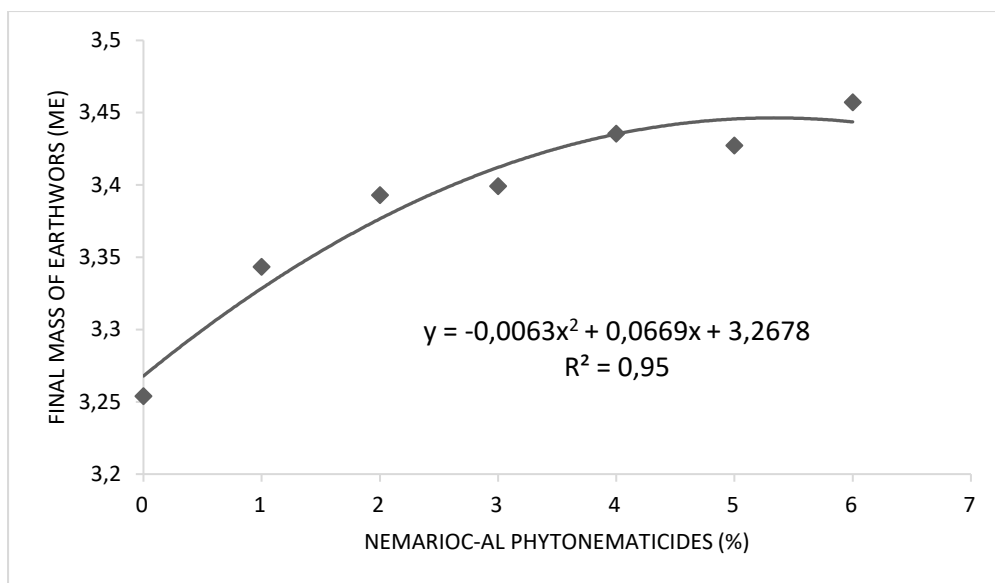


Fig 4.14 Response of final mass of earthworms (ME) to increasing concentrations of Nemarioc-AL phytonematicide (n=72).

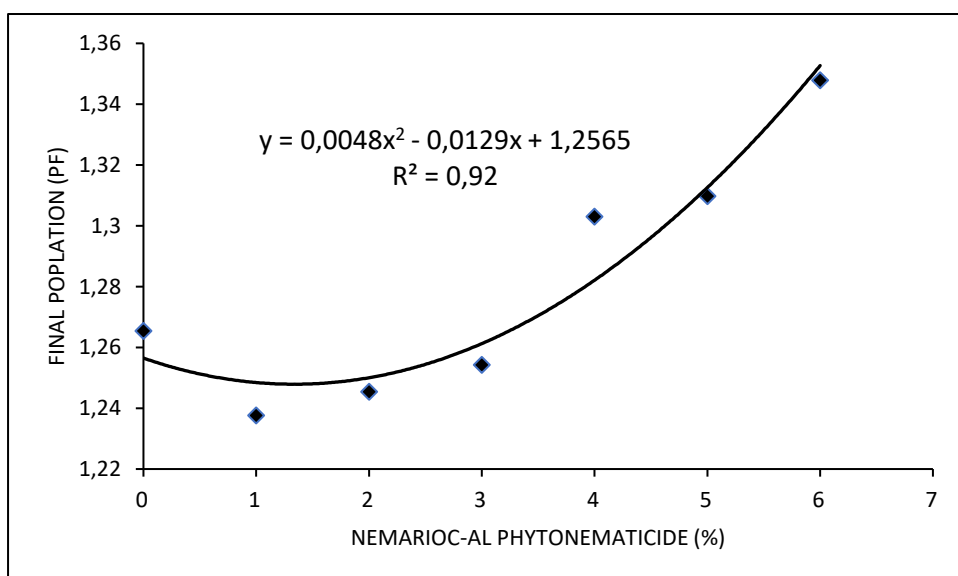


Fig 4.15 Response of final mass of earthworms (Pf) to increasing concentrations of Nemarioc-AL phytonematicide (n=72).

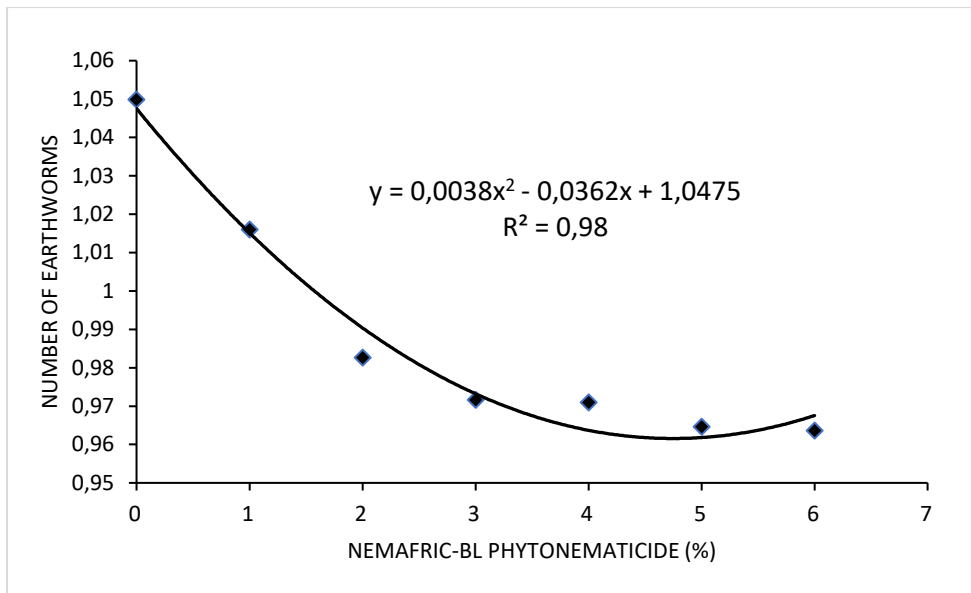


Fig 4.16 Response of number of earthworms (NW) to increasing concentrations of Nemafric-BL phytonematicide (n=72).

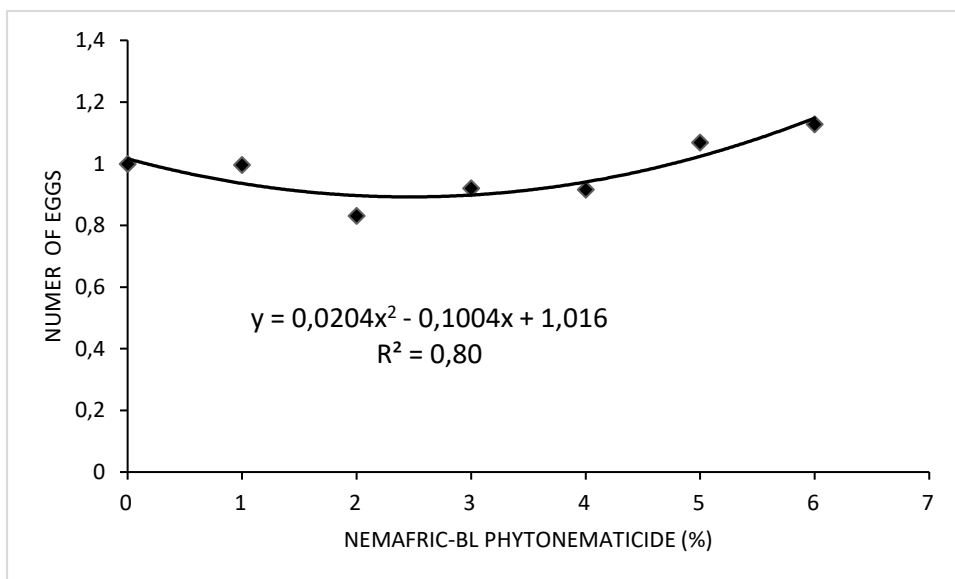


Fig 4.17 Response of number of eggs (NE) to increasing concentrations of Nemafric-BL phytonematicide (n=72).

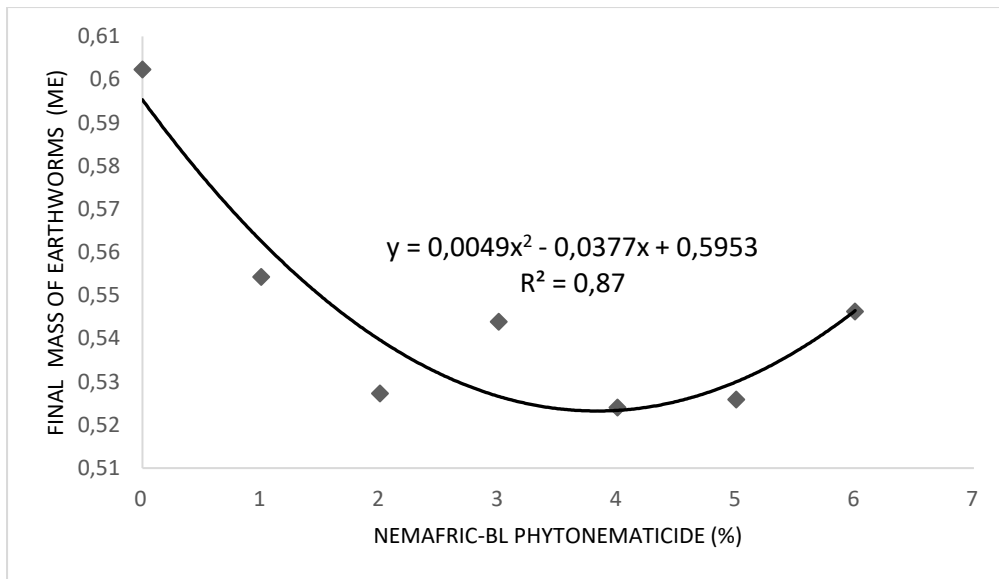


Fig 4.18 Response of number of final mass of earthworms (Pf) to increasing concentrations of Nemafric-BL phytonematicide (n=72).

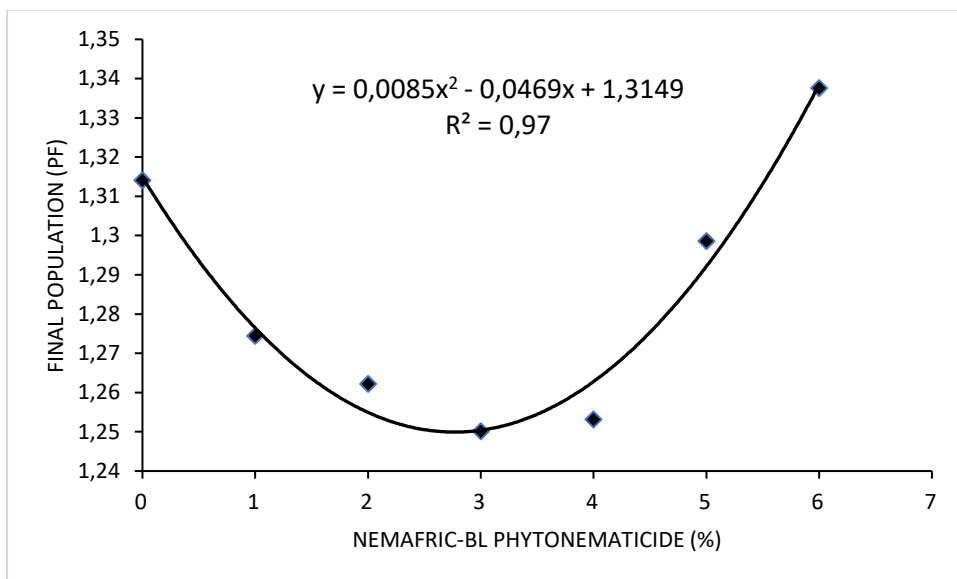


Fig 4.19 Response of number of final population (Pf) to increasing concentrations of Nemafric-BL phytonematicide (n=72).

The effects of increasing concentrations of Nemafric-BL phytonematicide on number of earthworms (NW), number of eggs (NE), final mass of earthworms (ME) and final population (Pf) exhibited positive or negative quadratic relations with model of the respective variables explained 0.95, 0.76, 0.87 and 0.97% coefficient of determination, respectively. Number of earthworms, ME and Pf exhibited negative quadratic relations at lower concentrations, while at higher concentration the respective earthworms' variables were increased. Reduction of respective variables shows that the Nemafric-phytonematicide at lower concentration levels was toxic to earthworms. However, at high concentration level was non-toxic to earthworms. These results confirm findings that Nemafric-BL phytonematicides at lower concentration level was more toxic and suppressed nematode numbers (Tseke and Mashela, 2018; Sithole *et al.*, 2016). Similar trends were observed on Swiss chard when Nemafric-BL phytonematicide was applied, nematodes population densities were decreased (Mashela *et al.*, 2021). However, Nemafric-BL phytonematicide stimulated cocoon production on earthworms, as observed on plant parasitic nematodes (Dube, 2016). Reduction of nematodes population densities was positive, however on earthworms' reduction was negative, since earthworms are non-target organisms. Therefore, Nemafric-BL phytonematicide had shown to be more sensitive to earthworms than Nemarioc-AL phytonematicide as with an optimal value of 3.46% while Nemarioc-AL had an optimal value of 9.46% (Table 4.3).

Table 4.3 Optimisation model for number of eggs (NE), number of earthworms (NEW), mass of earthworms (ME) and final population (Pf) in tomato plants as affected by increasing concentration of Nemarioc-AL and Nemafric-BL phytonematicides under greenhouse conditions.

	Model	R ²	x ²
Earthworms	Nemarioc-AL phytonematicide		
Number of eggs	$y = 0.0062x^2 - 0.0109x + 0.9207$	0.76	0.88
Number of earthworms	$y = -0.0003x^2 + 0.0153x + 0.9411$	0.95	25.5
Mass of earthworms	$y = -0.0063x^2 + 0.0669x + 3.2678$	0.95	5.31
Final population	$y = 0.0048x^2 - 0.0129x + 1.2565$	0.92	6.96
	Nemafric-BL Phytonematicide		
NE	$y = 0.0204x^2 - 0.1004x + 1.016$	0.86	2.46
NEW	$y = 0.0038x^2 + 0.0362x + 1.0475$	0.90	4.76
ME	$y = 0.0049x^2 - 0.0377x + 0.5953$	0.87	3.85
Pf	$y = 0.0085x^2 + 0.0469x + 1.3149$	0.97	2.76
Nemarioc-AL phytonematicide $X = -b_1/2b_2 = 9.66$			
Nemafric-BL phytonematicide $X = -b_1/2b_2 = 3.46$			

4.3.3 Proteomics

Sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) images showed clear and distinct protein bands to increasing concentrations of Nemarioc-AL phytonematicide with different molecular weights. The application of Nemarioc-AL phytonematicide at 2-4%, resulted in shallow bands indicating protein expression was downregulated. In contrast, increasing concentration of Nemarioc-AL phytonematicide at 8-64% intensified the protein expression showing upregulation. The identified band is from 30-40 kDa with molecular weight of 33 kDa has shown shallow appearances at 2-4%, and intensified as the concentrations of the phytonematicide increases. Other bands were observed between 40-50 kDa and 100-130 kDa with a molecular weight of 45 and 130 kDa, respectively. A non-intensified bands indicate a less toxic is a product to earthworms as evidenced by protein expression. This could be that the

earthworms were less stressed due to exposure of the phytonematicide at 2-4%, they activated some defence mechanism that withhold protein synthesis to conserve energy. This indicates that the phytonematicide has altered the protein synthesis at lower concentration levels as indicated by down-regulation of protein synthesis. As compared to control, protein synthesis under control remained stable or normal.

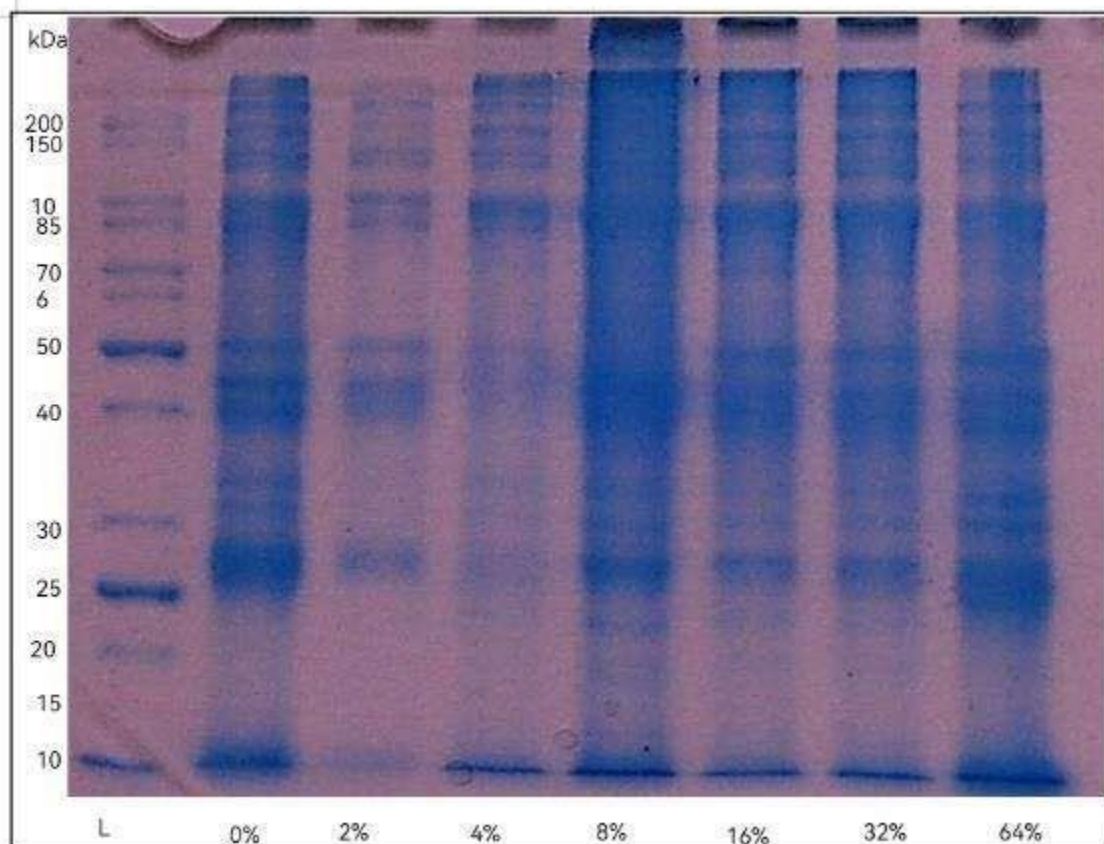


Fig 4.20 The effects of Nemarioc-AL phytonematicide on proteins of *Eisenia felita*.

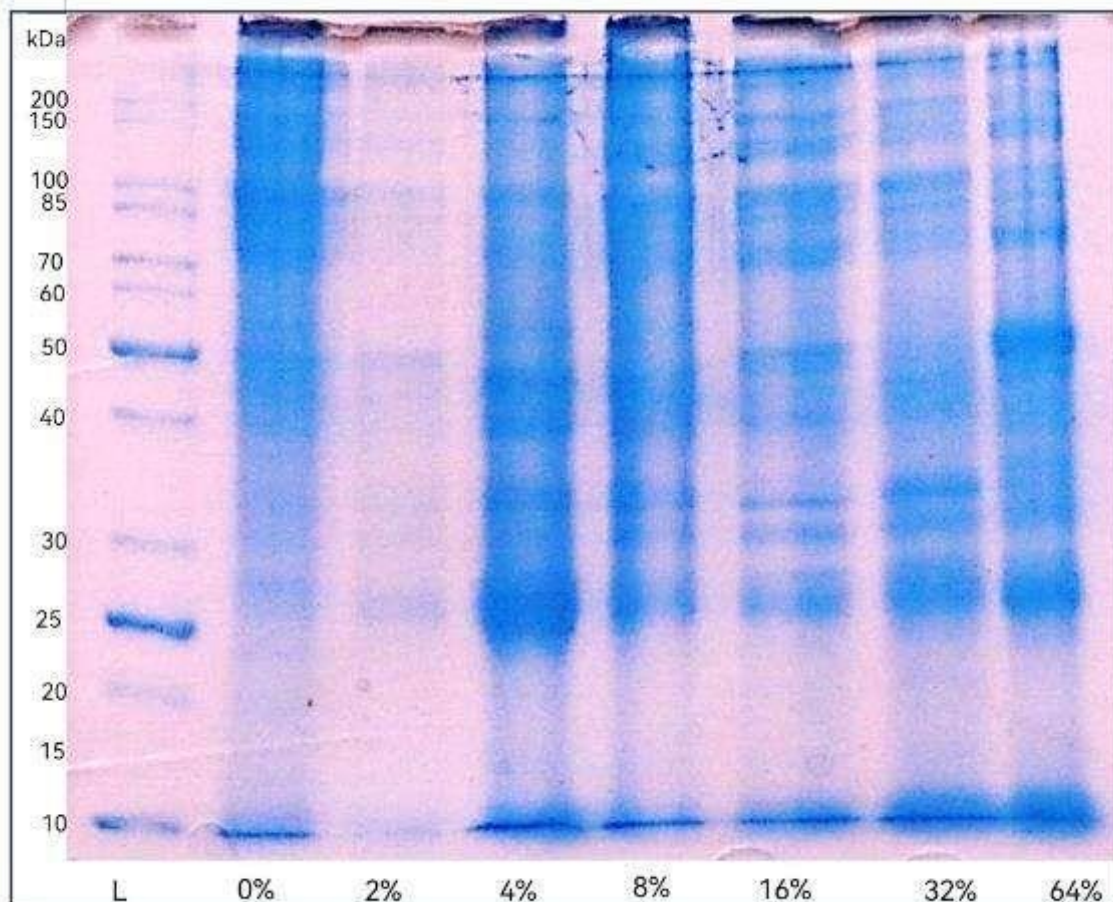


Fig 4.21 The effects of Nemafric-BL phytonematicide on proteins of *Eisenia felita*.

Protein expression can assist us understand adaptation of earthworm exposed to increasing concentrations of Nemafric-BL phytonematicide. The proteins bands identified were 30-40, 40-50 and 100-150 kDa with the molecular mass of 33, 45 and 130 kDa, respectively. Protein expression was downregulated, as shown by the shallow bands produced by the 2% application of the Nemafric-BL phytonematicide. In contrast, application of this product intensified protein expression at 4-64%, showing that proteins were upregulated. The increasing concentration of Nemafric-BL phytonematicide went beyond the worm's capacity for adaptation, inducing toxicity. In response to that, earthworms increased protein synthesis which are involved in stress,

detoxification and cell repairs. These proteins could be cytoplasmic chaperones, heat shock proteins, and antioxidant enzymes. Additionally, higher concentrations of phytonematicide may stimulate enzymes such as cytochrome P450, which play a role in phase I and II metabolic pathways or detoxification processes. Stronger or more intense bands on the gel would indicate that these proteins are up-regulated. These findings align with Kumar *et al.* (2011), who reported that the production or development of novel proteins, or the enhanced production of existing proteins that are produced solely under stressed conditions, is attributable to the stress.

4.5 Conclusion

The current study's response of plant variables, earthworms, and proteins followed a density-dependent growth pattern that showed stimulation at lower concentration levels, indicating no toxicity and inhibition at higher concentrations, showing toxicity to plants, earthworms, and protein synthesis. Therefore, lower concentration levels of Nemarioc-AL and Nemafric-BL phytonematicides should be used for plant protection as they demonstrated to be safe on living entities.

4.5 References

- Bango, H., Shadung, K.G. and P.W. Mashela. 2019. Influence of Nemarioc-AL and Nemafric-BL phytonematicides on mineral nutrient elements in tomato leaf tissues under field conditions. *Research on Crops* 20: 590-595.
- De Wandeler, H., Sousa-Silva, R., Ampoorter, E., Bruelheide, H., Carnol, M., Dawud, S.M., Dănilă, G., Finer, L., Hättenschwiler, S., Hermy, M. and B. Jaroszewicz, 2016. Drivers of earthworm incidence and abundance across European forests. *Soil Biology and Biochemistry* 99:167-178.

- Devi, P.I., Thomas, J. and Raju, R.K., 2017. Pesticide consumption in India: A spatiotemporal analysis. *Agricultural Economics Research Review* 30:163-172.
- Dube, Z.P. 2016. Nemarioc-AL and Nemafric-BL phytonematicides: Bioactivities in *Meloidogyne incognita*, tomato crop, soil type and organic matter. PhD Thesis, University of Limpopo, Sovenga, South Africa.
- Kamdem, M., Otomo, P.V., Ngakou, A. and N.N. Yanou, 2018. Distribution and diversity of earthworm (*Annelida, Clitellata*) populations across four land use types in northern Cameroon. *Turkish Journal of Zoology* 42:79-89.
- Kumar, A., Pandey, A.K., Singh, S.S., Shanker, R. and A. Dhawan. 2011. Cellular uptake and mutagenic potential of metal oxide nanoparticles in bacterial cells. *Chemosphere* 83:1124-1132.
- Liu, D.L., Johnson, I.R. and J.V. Lovett. 2003. Mathematical modelling of allelopathy. III. A model for Curve-fitting Allelochemical Dose responses. *Non- Linearity in Biology, Toxicology and Medicine* 1:37-50.
- Madaure, J.T., Mashela, P.W. and D.D. Waele, 2017. Responses of entomopathogenic nematode, *Steinernema feltiae* to cucurbitacin-containing Nemafric-BL phytonematicide. *Research on Crops* 18:739-744.
- Mafeo, T.P. and P.W. Mashela. 2010. Allelopathic inhibition of seedling emergence in dicotyledonous crops by *Cucumis* bio-nematicide. *African Journal of Biotechnology* 9:8349–8354.
- Mafeo, T.P., Mashela, P.W., Mphosi, M.S. and K.M. Pofu. 2011b. Modelling responses of maize, millet and sorghum seedlings to crude extracts of *Cucumis myriocarpus* fruit as pre-emergent bio-nematicide. *African Journal of Agricultural Research* 6:3678–3684.

- Malatji, K.P. 2017. Development of Mean Concentration Stimulation Point for fermented *Lantana camara* phytonematicide on tomato production. Mini-dissertation, University of Limpopo, Sovenga, South Africa.
- Malebe, A.L. 2019. Potential cucurbitacin chemical residues and non-phytotoxic concentration of two phytonematicide formulations in nightshade. Mini-dissertation, University of Limpopo, Sovenga, South Africa.
- Mashela, P.W, Pofu, K.M. and A.R. Ndhlala, 2024. Nemafric-BL Phytonematicide Induces Isoprenylation and Farnesylation in Guava Root-Knot Nematode, *Meloidogyne enterolobii*: Mechanism of Cucurbitacin-Cuticle Interaction.
- Mashela, P.W., D. De Waele, Z. Dube, M.C. Khosa, K.M. Pofu, G. Tefu, M.S. Daneel, and H. Fourie. 2017. Alternative nematode management strategies, p. 151-181. In: Fourie, H., V.W. Spaull, R.K. Jones, M.S. Daneel, and D. De Waele (eds.). Nematology in South Africa: A view from the 21st Century. Springer International Publishers: Heidelberg, Switzerland.
- Mashela, S.T., Shadung, K.G., Mafeo, T.P. and Mashela, P.W., 2021. Non-phytotoxic concentration of cucurbitacin containing phytonematicides on Swiss chard (*Beta vulgaris* var. Cicla) under greenhouse conditions. *Research on Crops* 22:327-333.
- Pelinganga, O.M. 2013. Developing phytonematicides using indigenous *Cucumis africanus* and *Cucumis myriocarpus* fruits for tomato production system. PhD Thesis, University of Limpopo, Sovenga, South Africa.
- Sithole, N.T., Pofu, K.M., Mashela, P.W., Dube, Z.P. and H. Araya. 2016. Overall sensitivity of *Pelargonium sidoides* and root-knot nematodes to Nemarioc-AL phytonematicide. *Transylvanian Review* 11:2996-3001.

- Tseke, P.E. and Mashela, P.W., 2018. Efficacy of fresh fruit from *Cucumis myriocarpus* as Nemarioc-AL phytonematicide on suppression of root-knot nematodes in tomato plant production. *Acta Agriculturae Scandinavica, Section B—Soil and Plant Science*, 68:161-165.
- Wang, X., Chang, L., Wang, G., Sun, Z., Ma, H., Sun, Q. and Li. 2010. Protein extraction from the earthworm *Eisenia fetida* for 2-DE. *Proteomics*. 10:1095-1099.
- Yatoo, A.M., Ali, M.N., Zaheen, Z., Baba, Z.A., Ali, S., Rasool, S., Sheikh, T.A., Sillanpää, M., Gupta, P.K., Hamid, B. and B. Hamid, 2022. Assessment of pesticide toxicity on earthworms using multiple biomarkers: a review. *Environmental Chemistry Letters*, 20:2573-2596.
- Zhao, H., Shi, S., Zhao, H., Guo, J., Yang, Z., Gao, H. and F. Lu, 2019. Proteomic analysis of the earthworm *Eisenia fetida* exposed to oxytetracycline in soil. *Royal Society of Chemistry* 9:41628-41638.

CHARPTER 5

RESPONSE OF RAW 264.7 MACROPHAGE CELLS TO NEMARIOC-AL AND NEMAFRIC-BL PHYTONEMATICIDES UNDER *IN VITRO* CONDITIONS: CELL VIABILITY TO MEASURE TOXICITY

Abstract

Nemarioc-AL and Nemafric-BL phytonematicides viewed as a future bionematicide for management of plant parasitic nematodes. As biopesticides, they are environmentally friendly over synthetic chemical nematicides. However, allelopathic effects of these botanicals can induce cytotoxicity on living entities. The objective of this study was to determine whether Nemarioc-AL and Nemafric-BL phytonematicides would have cytotoxic effects on Raw 264.7 macrophage cells. Approximately, 100 μ l of the cell cultures were seeded in 96-well microplates at 5×10^5 cells per well overnight, treated by Nemarioc-AL phytonematicide and Nemafric-BL phytonematicide then incubated for 24 hrs. Nemarioc-AL and Nemafric-BL phytonematicides at lower concentration level 1.25 to 0.078 mg/mL, stimulated cell viability, while at higher concentration levels 5 to 10 mg/mL inhibited cell viability on Raw 264,7 cell line. The stated products if detected in produce at higher concentration level, they can be cytotoxic to cells.

Keywords: MTT, Toxicity, Macrophages, Cell viability, Phytonematicide, Biopesticide

5.1 Introduction

The fact that Nemarioc-AL and Nemafric-BL phytonematicides are Botanicals natural products, they are therefore, assumed to be safe for humans since they are non-toxic to non-target organisms (Mashela *et al.*, 2024). Moreover, they are thought to be environment friendly as they are biodegradable, along with having advantages over synthetic nematicides in terms of cost, as treatment used to manage plant parasitic nematodes (Mashela *et al.*, 2017). However, Nemarioc-AL and Nemafric-BL phytonematicides have faced issues with phytotoxicity, a common challenge in botanicals due to their reliance on allelochemicals as bioactive compounds with a potential to induce toxicity (Sithole, 2016). Given the common instances of cytotoxicity associated with the use of botanicals in traditional medicine (Kamanja *et al.*, 2018), it's essential to study secondary plant metabolites before their application. Information on the viability of living and dead cells following exposure to Nemarioc-AL and Nemafric-BL phytonematicides would be obtained using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide) assay (Ghasemi *et al.*, 2021). The term "cell cytotoxicity" describes the capacity of specific substances to kill live cells (Celik, 2018). A test product, derived from plant extracts might first be subjected to cytotoxicity tests to assess its usage threshold (Celik, 2018).

Bioactive compounds and chemical compounds are regarded as cytotoxic only if they hinder essential metabolic functions, resulting in abnormal disorders in living entities leading to perversion behavior or death (Kamanja *et al.*, 2018). The death could be through necrosis, a condition in which the membrane integrity is compromised and uncontrollable death occur or a programmed cell death (apoptosis) (Istifli *et al.*, 2019). Thus, if products were not tested humans can be exposed to a broad range of foreign

chemicals, which often referred to as "xenobiotics" collectively. These substances include natural compounds found in plant food as well as artificial compounds found in plant produce, medications and environmental contaminants (León-Mejía *et al.*, 2021). According to Dhakal *et al.* (2018), Godheja *et al.* (2018), Miglani *et al.* (2022) xenobiotics, which include pesticides used in plant protection, azodyes, phenolics, polycyclic aromatic hydrocarbons (PAHs), halogenated compounds, personal care products (PCPs), pharmaceutical active compounds (PhACs), nitroaromatic compounds, triazines, and chlorinated compounds, have a long-term persistence and slow or nonexistent biodegradation in ecosystems, causing adverse environmental effects. Hence, negatively affecting the wellbeing of living entities.

Although many bioactive compounds have therapeutic or pests-repelling benefits for human health and plant protection, their cytotoxic qualities can also lead to a variety of health problems (Rasool *et al.*, 2023). Rasool *et al.* (2023) added that although these bioactive compounds are utilized as anticytotoxic medicines, they also have certain cytotoxic effects on human life and other organisms. Plants possess plethora of bioactive compounds commonly known as secondary metabolites. These bioactive compounds are a class of molecules found in the bodies of fauna, flora, and microorganisms (Setyorini and Antarlina, 2022). They are produced by secondary metabolic processes from a large number of primary metabolite components (Setyorini and Antarlina, 2022). The bioactive compounds have allelopathic effects, which can inhibit growth of other living entities (Šoln *et al.*, 2022). It's necessary to test cell viability when exposed to bioactive substances because some plant species are more toxic than others. Therefore, the MTT assays would be used to assess level of toxicity induced by Nemarioc-AL and Nemafric-BL phytoneaticides. The objective of this study

was to determine whether Nemarioc-AL and Nemafric-BL phytonematicides would have cytotoxic effects on Raw 264.7 macrophage cells.

5.2 Materials and Methods

The study was conducted at Biochemistry laboratory, University of Limpopo, South Africa (23°53'10"S, 29°44'15"E). The study was initiated from spring (August) 2024. The MTT assay was technically named after a dye known as Tetrazolium (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide). It is used to assess cytotoxicity of cells, to determine whether the cells are viable or not when exposed to increasing concentrations of tested compounds (Kuate *et al.*, 2017). The MTT is a cell permeant as a result of positive and lipophilic structure, the MTT reagent normally passes the cell membrane and mitochondrial inner membrane of viable cells (Ghasemi *et al.*, 2021). It is reduced to a water insoluble formazan by metabolically active cells. To assess cytotoxicity using MTT assay, the mitochondria of cells must be healthy, and the nicotinamide adenine dinucleotide phosphate hydrogen (NADPH) dependent oxidoreductase enzyme is then produced only when a cell is viable. If cells are viable, the enzyme turns tetrazolium dye to be purple in color. The purple-colored compounds are known as formazan. Thus, the chromogenic nature of redox reactions allows colorimetric measurement of intracellular formazan production, enabling analysis of plate reader data through spectrophotometry.

Raw 264.7 macrophage cells were bought from ATCC and stored in a -80 °C freezer until required. The cell lines were thawed, suspended and cultured in a Dulbecco's Modified Eagle Medium (DMEM) supplemented with 1 x PSN and 10% fetal bovine

serum (FBS) within T75 culture flask. Cells were allowed to regenerate or replicate itself for period of a week in a CO₂ incubator at 37 °C and 5% CO₂. The medium was removed and the remaining cells attached on the flask were detached by cell scraper. Cell counting was done using 1:1 cell to trypan-blue dye ratio and cell Countess (Thermofisher, USA) was used to count cells.

5.2.1 Experimental Design

Two samples, Nemarioc-AL phytonematicide and Nemafric-BL phytonematicide each were placed in serial dilution of 0, 0.812, 1.625, 3.25, 6.5, 12.5, 25, 50 and 100 mg/mL in microplate well. Treatments were arranged in completely randomized design (CRD), replicated 3 times. Curcumin was used as a standard at 50 and 100 µM in the current study.

5.2.2 Procedure

In order to examine the toxic levels of the Nemarioc-AL and Nemafric-BL phytonematicides, 100 µl of the cell cultures were seeded in 96-well microplates at 5 x10⁵ cells per well overnight and followed treatment with various concentration of Nemarioc-AL and Nemafric-BL phytonematicides for 24 hrs. After 24 hrs of treatment, just before the addition of 5 mg/mL of MTT dye, cell morphology pictures were captured with microscope (Makola *et al.*, 2020), thereafter MTT was added to each well and then microplates were incubated at 37 °C in 5% CO₂ incubator for 2 hrs. The formed formazan products were dissolved with 2% HCl acidified isopropanol for 10 min. Thereafter absorbance was measured using GloMax-Multi microplate reader (Promega, USA) at 560 nm. Percentage viability was calculated using this formula:

$$\text{Cell viability (\%)} = \frac{\text{Average OD (experimental group)}}{\text{Average OD (untreated group)}} \times 100$$

5.2.3 Data analysis

Data for concentration response curves were analysed by GraphPad Prism® version 5.2, GraphPad Software Inc., San Diego, CA, USA (Motulsky, 2007).

5.3 Results and Discussion

The MTT assay was used to assess cell viability post treatment with different concentrations in order to assess the cytotoxicity profiles of Nemarioc-AL and Nemafric-BL phytonematicides on murine Raw 264.7 macrophages. The increasing concentration of Nemarioc-AL phytonematicide exhibited 50% cytotoxic effects to cell viability of Raw 264,7 cell line at 5 and 10 mg/mL relative to untreated and Curcumin treated control (Figure:2 A). However, Nemafric-BL phytonematicides, showed 50% cytotoxicity at 5 and 10 mg/mL. As the concentrations of Nemarioc-AL and Nemafric-BL phytonematicides decreased from 1.25 to 0.078 mg/mL, the cell viability of Raw 264.7 cell lines were stimulated relative to positive control (Figure: 5.1A and 5.2A).

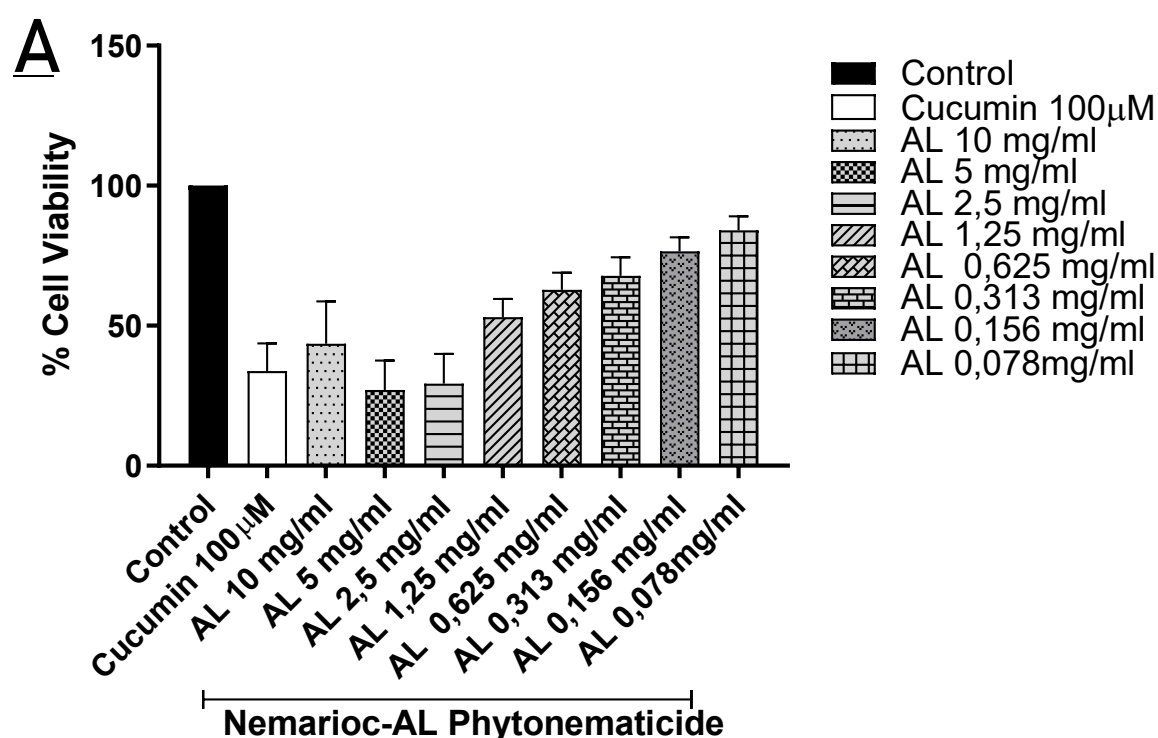
The observed Raw 264.7 cell viability results, show that increasing concentrations of Nemarioc-AL and Nemafric-BL phytonematicides has a dose-response relationship. The higher concentrations of Nemarioc-AL and Nemafric-BL phytonematicides had a negative effect on cells thereby inducing toxicity to cells. Cell morphology imaging showed apoptotic bodies, at 10 and 5 mg/mL of both the phytonematicides as in curcumin control (Figure 5.1B and 5.2B). The Nemarioc-AL phytonematicide showed circular shaped Raw 264.7 cells from 2.5 to 0.313 mg/mL, however, one would notice that the size of the circular cells shape increases with decreasing Nemarioc-AL

phytonematicide concentration (Figure 5.1B and 5.2B). Moreover, at 0.136 and 0.078 mg/mL the cells have retained their spindled shaped morphology and at 0.078 mg/mL shows that cells have doubled, as there are claps of small circular cells (Figure 5.1B). While focusing on cell morphology, Nemafric-BL phytonematicide showed similar patterns, however, the cells treated with 2.5 and 1.25 mg/mL Nemafric-BL are circular with increasing in size on the lower concentration. Moreover, 0.625 and 0.313 cells attained their spindle shaped morphology while at 0.136 and 0.078 mg/mL cell have divided as there is number of daughter cells (Figure 5.2B) (Fukuyama *et al.*, 2022). The reduced cell viability of Raw 264,7 cells at 10, 5 and 2.5 mg/mL means the products contains bioactive compound known as cucurbitacin A and B, with a potential to induce cytotoxicity at higher concentrations on living cells, it had negative impact on the living cells at a stated concentration level (Chen *et al.*, 2005).

It is suggested that Cucurbitacin A has induced disruption of the cell membrane and interfered with the metabolic activities of cells, causing death. In this regard, it means the cells have undergone sufficient stress and then cell death, leading to disruption of mitosis (cell division) (Istifli *et al.*, 2019). At the high concentration level, treatments inhibited the growth of cells and duplication of DNA, so that cells cannot go to the next stage of cell division known as the prophase. That is the reason why cells were unable to cleave sufficient amount of tetrazolium dye and form formazan. Hence an indication of cytotoxicity of the products at these concentration level.

The observed results agree with the findings that different plant extracts from *Acmella ciliata* (Kunth) Cass., *Coriandrum sativum* L., and *Glebionis coronaria* L. at higher concentration levels (320 to 640 µg/mL) induced mortality on Vero cells tested by MTT

assay (Chan *et al.*, 2015). Hence, resulted in reduced cell viability. At lower concentrations, the cell viability 1.25 to 0.078 mg/mL treated by Nemarioc-AL and Nemafric-BL phytonematicides have shown no signs of cytotoxicity to cells. At these concentrations level, cell viability was higher, indicating that cell division can still occur leading to cell proliferation. These confirm observations that plant extracts from *Andrographis paniculata* (Burm.f.) Wall. ex Nees and *Andrographis paniculata* over time (24 to 48 hrs) at different concentration levels (4—500 µg/mL) were not cytotoxic to murine hepatocytes, thymocytes, and splenocytes cell lines (Ala *et al.*, 2018). Therefore, the higher concentration level of Nemarioc-AL and Nemafric-BL phytonematicides in this study have shown to inhibit cell viability on murine Raw 264.7 macrophages. Hence, signs of cytotoxicity were observed at lower concentration levels.



B

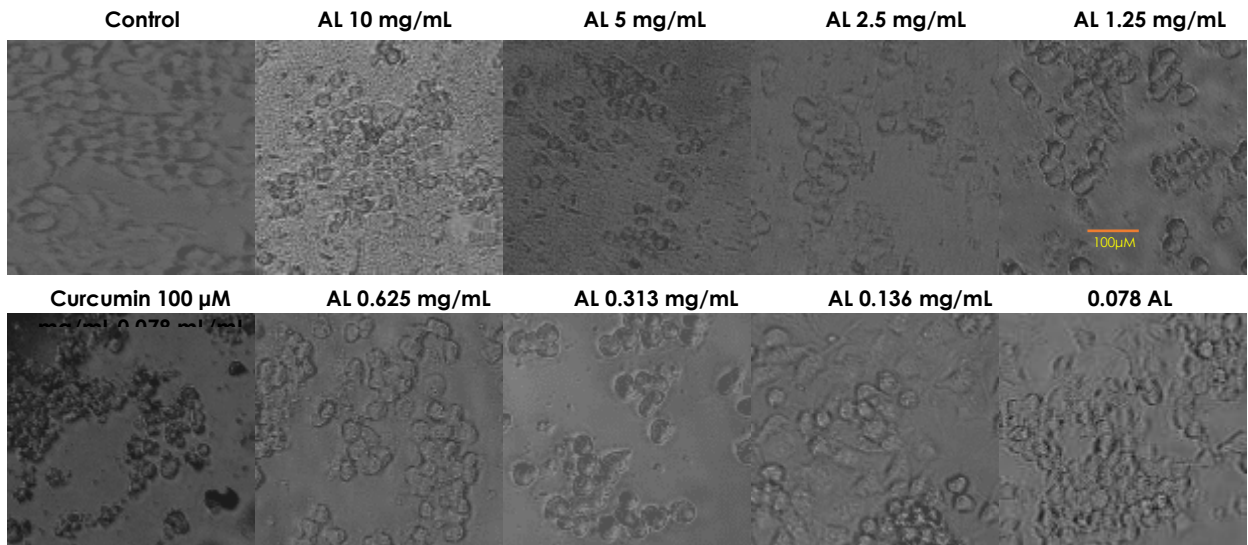
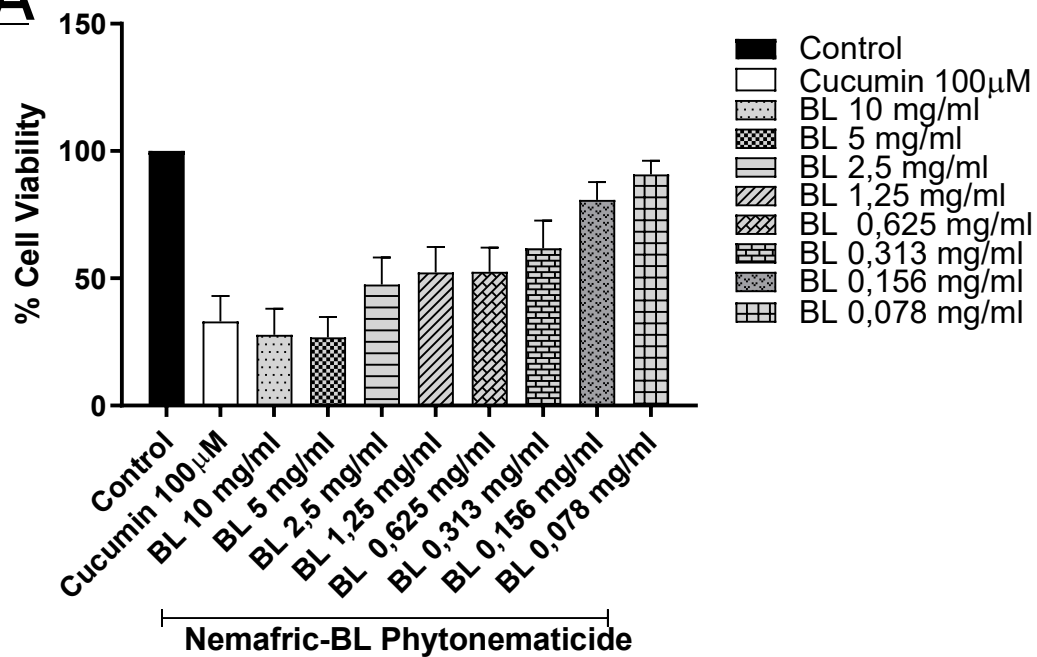


Fig 5.1 A. The effects of Nemarioc-AL phytonematicide on cell viability of murine Raw 264.7 macrophages under *in vitro* conditions. B. Apoptotic effects caused by Nemarioc-AL phytonematicide on murine Raw 264.7 macrophages as observed under a microscope.

A



B

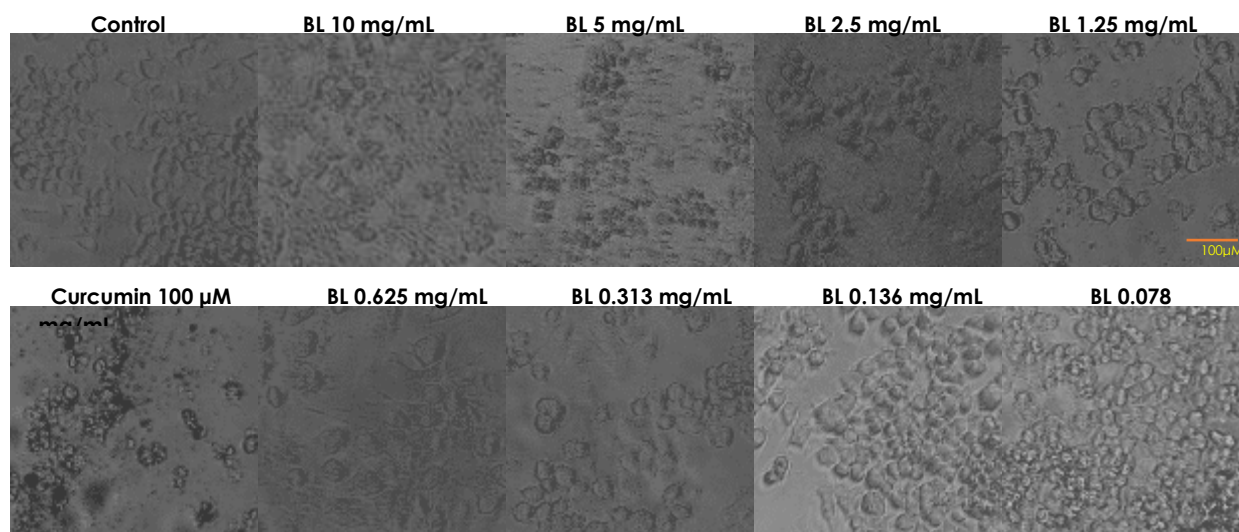


Fig 5.2 A. The effects of Nemafric-BL phytonematicide on murine Raw 264.7 macrophages under *in vitro* conditions. B. Apoptotic effects of Nemafric-BL phytonematicide on murine Raw 264.7 macrophages as observed under a microscope.

5.4 Conclusion

The current study has shown that Nemarioc-AL and Nemafric-BL phytonematicides were non-cytotoxic at the developed concentration range of 1.25 to 0.078 mg/mL on murine Raw 264.7 macrophages. The concentration above this depicted cytotoxic to the cells. Therefore, any concentration above 2,5 mg/mL in can be detrimental to human beings and animals. Therefore, farmers should adhere the developed concentrations in produce for safety purposes of the two products.

5.5 References

- Ala, A.A., Olotu, B.B. and C.M.D Ohia. 2018. Assessment of cytotoxicity of leaf extracts of *Andrographis paniculata* and *Aspilia africana* on murine cells *in vitro*. *Archives of basic and applied medicine* 6:1-13.
- Celik, T.A. ed., 2018 Introductory Chapter: Cytotoxicity. Chapter, 1: 1-5. IntechOpen (July 25th, 2018), 10.5772/intechopen.77244

- Chan, S.M.; Khoo, K.S.; and N.W. Sit, 2015. Interactions between plant extracts and cell viability indicators during cytotoxicity testing: Implications for ethnopharmacological studies. *Tropical Journal of Pharmaceutical Research* 14:1-9.
- Chen, J.C., Chiu, M.H., Nie, R.L., Cordell, G.A. and S.X. Qiu. 2005. The cucurbitacins and cucurbitane glycosides: Structures and biological activities. *Natural Products Report* 22:386-399.
- Dhakal, K., Gadupudi, G.S., LehmLer, H.J., Ludewig, G., Duffel, M.W. and L.W. Robertson. 2018. Sources and toxicities of phenolic polychlorinated biphenyls (OH-PCBs). *Environmental Science and Pollution Research* 25:16277-16290.
- Fukuyama, T., Yan, L., Tanaka, M., Yamaoka, M., Saito, K., Ti, S.C., Liao, C.C., Hsia, K.C., Maeda, Y.T. and Y. Shimamoto. 2022. Morphological growth dynamics, mechanical stability, and active microtubule mechanics underlying spindle self-organization. *Proceedings of the National Academy of Sciences* 19:1-11.
- Ghasemi, M., Turnbull, T., Sebastian, S. and I. Kempson. 2021. The MTT assay: utility, limitations, pitfalls, and interpretation in bulk and single-cell analysis. *International Journal of Molecular Sciences* 22:1-30.
- Godheja, J., Shekhar, S.K., Siddiqui, S.A. and D.R. Modi. 2016. Xenobiotic compounds present in soil and water: a review on remediation strategies. *Journal of Environmental and Analytical Toxicology* 6:2161-0525.
- Istifli, E.S., Hüsnet, M.T. and H.B. İla. 2019. Cell division, cytotoxicity, and the assays used in the detection of cytotoxicity. Cytotoxicity-definition, identification, and cytotoxic compounds, *Intech. Open* 2019.

- Kamanja, I.T., Mbaria, J.M., Gathumbi, P.K., Mbaabu, M., Kabasa, J.D. and S.D. Kiama. 2018. Cytotoxicity of selected medicinal plants extracts using the brine shrimp lethality assay from Samburu County, Kenya. *Journal of Media Research* 4: 249-255
- Kuete, V., Karaosmanoğlu, O. and H. Sivas, 2017. Anticancer activities of African medicinal spices and vegetables. In Medicinal spices and vegetables from Africa (pp. 271-297). *Academic Press*.
- León-Mejía, G., Guevara, A.M., Moreno, O.F. and C.U. Cruz. 2021. Cytotoxicity as a Fundamental Response to Xenobiotics. Cytotoxicity-New Insights into Toxic Assessment, IntechOpen: London, United Kingdom
- Makola, R.T., Mbazima, V.G., Mokgotho, M.P., Gallicchio, V.S. and T.M. Matsebatlela. 2020. The Effect of Lithium on Inflammation-Associated Genes in Lipopolysaccharide-Activated Raw 264.7 Macrophages. *International Journal of Inflammation* 1: 1-18.
- Mashela, P.W, Pofu, K.M. and A.R. Ndhlala, 2024. Nemafric-BL Phytonematicide Induces Isoprenylation and Farnesylation in Guava Root-Knot Nematode, *Meloidogyne enterolobii*: Mechanism of Cucurbitacin-Cuticle Interaction.
- Mashela, P.W., D. De Waele, Z. Dube, M.C. Khosa, K.M. Pofu, G. Tefu, M.S. Daneel, and H. Fourie. 2017. Alternative nematode management strategies, p. 151-181. In: Fourie, H., V.W. Spaull, R.K. Jones, M.S. Daneel, and D. De Waele (eds.). *Nematology in South Africa: A view from the 21st Century*. Springer International Publishers: Heidelberg, Switzerland.

- Miglani, R., Parveen, N., Kumar, A., Ansari, M.A., Khanna, S., Rawat, G., Panda, A.K., Bisht, S.S., Upadhyay, J. and Ansari, M.N., 2022. Degradation of xenobiotic pollutants: an environmentally sustainable approach. *Metabolites* 12:818.
- Motulsky, H.J., 2007. Prism 5 statistics guide, 2007. GraphPad Software 31:39-42.
- Rasool, N., Omer, M.O., Javeed, A., Nawaz, M., Rasheed, M.A., Imran, M., Hussain, M., Dawood, A.F., Mushtaq, Z. and E. Al Jbawi. 2023. Phytochemical properties and *in-vitro* cytotoxicity, genotoxicity and mutagenicity assessment of ethanolic and aqueous extracts of *Argyrolobium roseum* (Camb.). *International Journal of Food Properties* 26:1457-1469.
- Sithole, N.T., Pofu, K.M., Mashela, P.W., Dube, Z.P. and H. Araya. 2016. Overall sensitivity of *Pelargonium sidoides* and root-knot nematodes to Nemarioc-AL phytonematicide. *Transylvanian Review* 11:2996-3001.
- Šoln, K., Klemenčič, M. and J.D. Koce. 2022. Plant cell responses to allelopathy: from oxidative stress to programmed cell death. *Protoplasma*, 259:1111-1124.

CHAPTER 6

THE EFFECTS OF NEMARIOC-AL AND NEMAFRIC-BL PHYTONEMATOCIDES ON EISENIA FETIDA UNDER IN VITRO CONDITIONS

Abstract

Earthworms play important role in maintaining soil productivity and often used as a standard for assessing toxicity in agroecosystem. They are one of the abundance creatures found in warm region and coexist with plant parasitic nematodes in the soil. Application of synthetic nematicides drastically reduced population of both nematodes and earthworms necessitating alternative management strategies such as the use of bionematicides in crop husbandry. The phytonematicides are regarded as toxic and used for plant protection purposes which can affect earthworms. The objective of the study was to determine structural deformity and toxicity of Nemarioc-AL and Nemafric-BL phytonematicides on *E. fetida* under in vitro conditions. Treatment constituted 0, 2, 4, 8, 16, 32 and 64% Nemarioc-AL or Nemafric-BL phytonematicide. Three earthworms were placed on a filter paper in a petri-dish. And soaked with, 10 mL each concentration of the stated phytonematicide. Nemarioc-AL and Nemafric-BL phytonematicides high concentrations of 8 to 64% induced mortality and inhibited viability up to 100% with more structural deformities. At lower concentrations less structural deformities were observed. Higher concentration phytonematicide of the have shown to be toxic to earthworms.

Keywords: Biodiversity, toxicology, pesticides, structural deformity, ecosystem

6.1 Introduction.

The intensification of agricultural practices, especially the widespread use of synthetic pesticides and fertilizers has resulted in a decline in the biodiversity of agroecosystem soils (Pelosi *et al.*, 2016). According to Alengebawy *et al.*, (2021), these synthetic compounds are often classified as anthropogenic sources which are toxic to non-target organisms like earthworms (*Eisenia fetida*). Earthworms are regarded as an essential microorganism, which directly or indirectly influence availability of macro and micro elements for plants uptake and maintain soil productivity. (Datta *et al.*, 2016). Earthworms are also used as standard for microorganism model for environmental risk assessment of toxic chemicals and soil toxicology (Miglani and Bisht, 2019). Furthermore, they are considered ecosystem engineers because they impact soil structure and chemistry, especially through the incorporation and fragmentation of organic matter, nutrient cycling, aggregate formation and soil porosity (Maréchal *et al.*, 2021). However, earthworms are negatively affected by pesticides used in plant protection.

Earthworms are abundance creatures which co-exists with plant parasitic nematodes in soil ecosystem which tends to be vulnerable to synthetic pesticides while nematode are managed (Dionísio *et al.*, 2018). Earthworms treated by synthetic chemical nematicides reduced the ability to complete the most critical and valuable functions in the soil (Jansirani *et al.*, 2012). The exposure of earthworms to capiscol, stratos ultra jardin, polyvalent, round-up GT plus and polyflor resulted in toxicity to the products (Ouina *et al.*, 2017). Furthermore, synthetic chemical pesticides can induce mortality on population densities of earthworms (Datta *et al.*, 2016). The use of synthetic pesticides has shown environmental unfriendly leading to the development of

biopesticides Nemarioc-AL and Nemafric-BL phytonematicides (Mashela *et al.*, 2017). Biopesticides developed for plant protection purposes are known to be toxic to a target pest without information on how the product will affect other microorganisms such as earthworms. Therefore, its imperative to assess safety of biopesticide products on non-target organisms to fully understand their effects. The objective of the study was to determine structural deformity and toxicity of Nemarioc-AL and Nemafric-BL phytonematicides on *Eisenia fetida* under *in vitro* conditions.

6.2 Materials and Methods

6. 2.1 Description of the study location

The research was conducted at the Green Biotechnologies Research Centre of Excellence (GBRCE), University of Limpopo, South Africa (23°53'10'S, 29°44'15'E). A temperature set for an incubator was 20 °C inside a Ethnopharmacology laboratory.

6.2.2 Treatments and Experimental Design

Treatments for each product Nemarioc-AL or Nemafric-BL phytonematicides comprised of 0, 2, 4, 8, 16, 32 and 64% arranged in a completely randomized design (CRD) replicated 3 times.

6.2.3 Procedures and Cultural Practices

Earthworms were collected from a growing media in cultured pods inside the greenhouse. Adult worms with the weight of 300 and 600 mg (*E. fetida*) and fully developed clitellum were used for all the experiments. Separate trials, for Nemarioc-AL and Nemafric-BL phytonematicides were conducted concurrently under *In vitro* conditions. Earthworms were collected manually and rinsed by distilled water. Three

earthworms were placed on a filter paper in a petri-dish. Approximately, 10 mL from 0, 2, 4, 8, 16, 32 and 64% Nemarioc-AL and Nemafric-BL phytonematicides concentrations were pipetted per petri-dish. Parafilm was used to seal petri-dishes containing the immersed species. The petri-dishes were kept in an incubator set at $25 \pm 2^{\circ}\text{C}$ for 24h.

6.2.4 Data Collection

The effects of Nemarioc-AL and Nemafric-BL phytonematicides were determined by using needle for viability and mortality while structural deformity was assessed by using pictures. The response of earthworms to a needle indicated that it was viable and non-response indicated mortality. Structural deformities were observed under a camera.

6.2.5 Data Analysis

Data for concentration response curves were analysed by GraphPad Prism® version 5.2, GraphPad Software Inc., San Diego, CA, USA (Motulsky, 2007).

6.3 Results and Discussion

6.3.1 Mortality and cell viability using Nemarioc-AL phytonematicide

The increasing concentrations of Nemarioc-AL phytonematicide at 2 to 8% doses on earthworm stimulated viability contributing 100%, and inhibited mortality to up to 0% (Figure 6.1). In contrast, a dose from 16 to 64% Nemarioc-AL phytonematicide inhibited viability on earthworms, while on the other side it induced mortality (Figure 6.1). The observed results suggests that exposure to earthworms at low concentration of Nemarioc-AL phytonematicide did not have toxic effects earthworms (Figure 6.3).

In contrast, higher concentrations were more toxic to earthworms as indicated by structural deformities (Figure 6.3). Earthworms exposed to toxic heavy metal in the soil, reduced mobility, reproduction, enzymes activities and growth were identified (Yadav *et al.*, 2023). The result in the current study confirm with findings where two pesticides cythion and dieldrin were tested for toxicity on *E. fetida* and that at low concentrations, earthworms were seen viable but at higher concentration levels, their body was melted, broken and destroyed (Shahzad and Pawar, 2017). These results on structural deformity were observed when *Eudrilus eugeniae* was exposed to malathion pesticide morphological abnormalities like lifting the body, coiling, and curling were observed in all individuals exposed to the highest concentrations for 24 h (Jeyaprakasam *et al.*, 2021). At higher concentration earthworms experienced coiling, release of coelomic fluid, redness, segmental constriction and swelling.

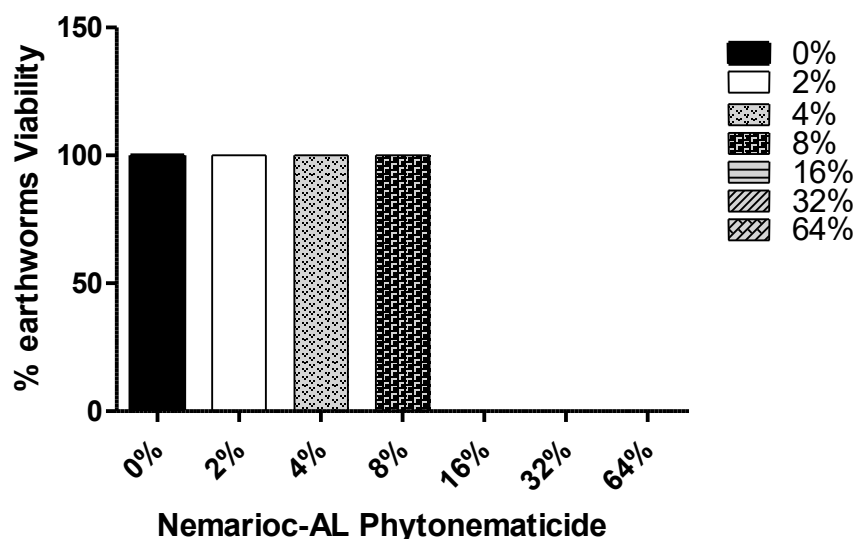


Fig 6.1 Response of *Eisenia fetida* on viability exposed to increasing concentrations of Nemarioc-AL phytonematicide under *in vitro* conditions

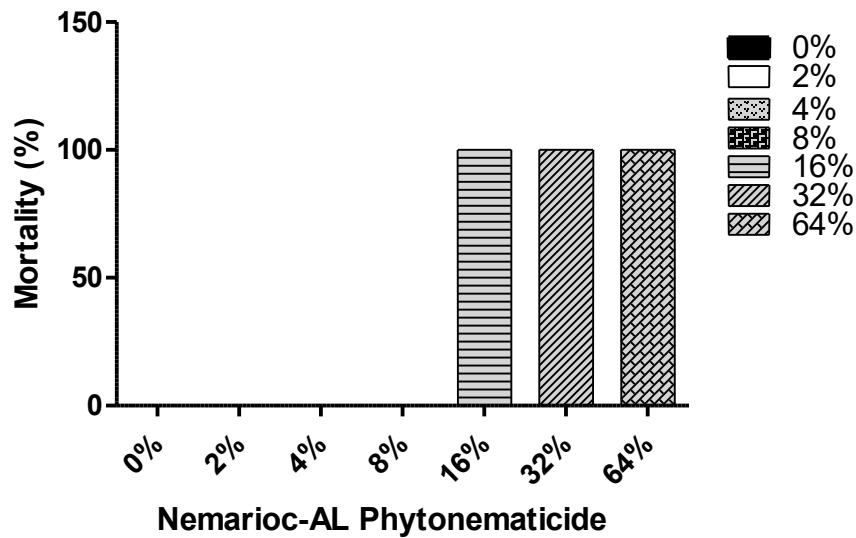


Fig 6.2 Response of *Eisenia fetida* on mortality exposed to increasing concentrations of Nemarioc-AL phytonematicide under *in vitro* conditions

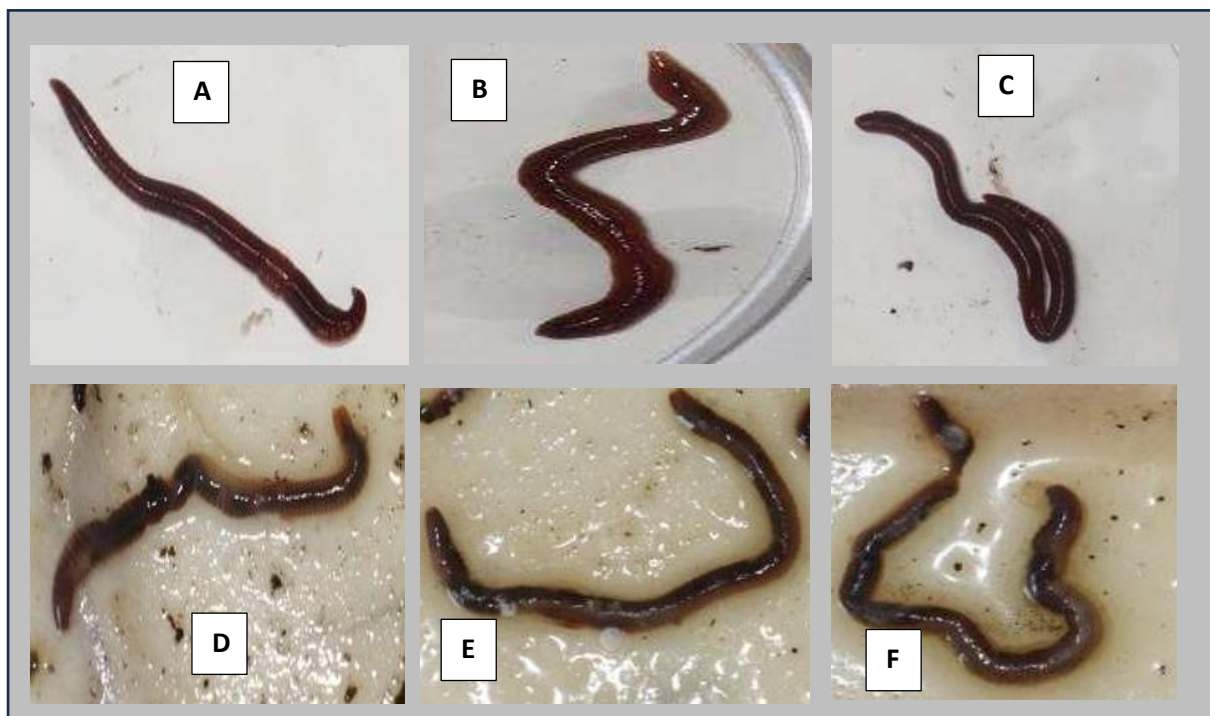


Fig 6.3 Structural deformities of *Eisenia fetida* on exposure to increasing concentrations of Nemarioc-AL phytonematicide under *in vitro* conditions at 24 h. A (Control – 0%), B – 4%, C - 8%, D 16%, E – 32% and F – 64%

6.3.2: Mortality and viability using Nemafric-BL phytonematicide

The response of earthworms exposed to increasing concentrations of Nemafric-BL phytonematicide have shown 100% viability at 2 to 8%. In contrast, at 16 to 64%, no viability was observed (Figure 6.4). Mortality induced by Nemafric-BL phytonematicide was observed at 16 to 64%, whereas no mortality rate observed at 0 to 8% (Figure 6.5). These results suggest that the phytonematicide did not have toxic effects on viability of earthworms at lower concentration levels, while at higher concentration levels toxicity was induced. At 4 and 8% even though earthworms were viable they were signs of deformities shrinking was observed. Relative to untreated control, there were no signs of shrinking observed. At 16, 32 and 64% there were bleeding or blood like secretions around the worm's depicting toxicity of a product to earthworms. The release of coelomic fluid (yellow color) was clearly observed on 64%. Swelling was observed on 16% and mortality was observed on 16, 32 and 64%. Lastly, they were signs of decay at high concentration level indicating that these earthworms died several hours before 24 h after incubation. These signs on structural deformities indicate that Nemafric-BL phytonematicide was toxic to earthworms. However, lower concentration levels did not show much signs of toxicity. When earthworms were used to assess toxicity of pesticides, same structural deformities were observed on earthworms (Yatoo *et al.*, 2022). On other plant extracts, commercial neem (*Azadirachta indica* A. Juss.) extracts had reduced the radial thickness of the epidermis, body wall and thickness of intestinal epithelium earthworms (*Pheretima peguana*) at high concentration level (Muangphra, and Gooneratne, 2011).

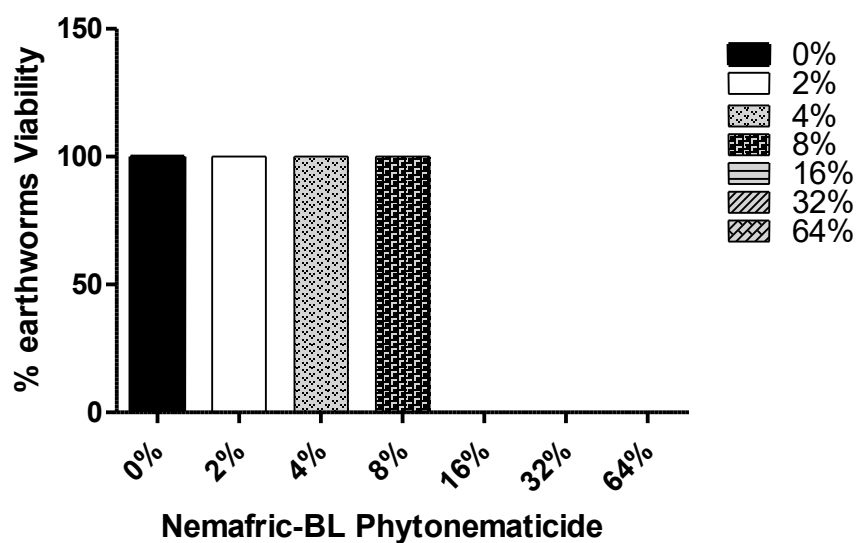


Fig 6.4: Response of *Eisenia fetida* on viability exposed to increasing concentrations of Nemafric-BL phytonematicide

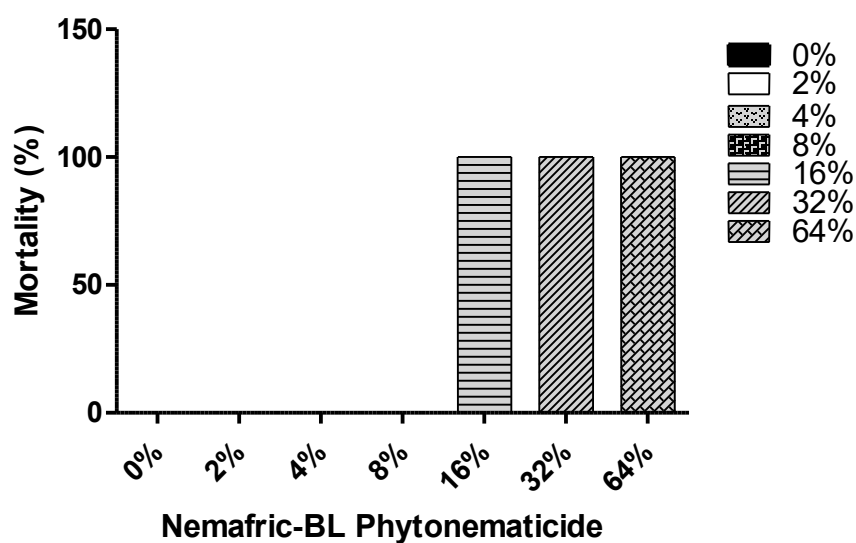


Fig 6.5: Response of *Eisenia fetida* on mortality exposed to increasing concentrations of Nemafric-BL phytonematicide under *in vitro* conditions.



Fig 6.6: Structural deformities of *Eisenia fetida* on exposure to increasing concentrations of Nemafric-BL phytonematicide under *in vitro* conditions at 24 h.

6.4: Conclusion

Nemarioc-AL and Nemafric-BL phytonematicides were non-toxic to earthworms at low concentrations with moderate structural deformities observed. These two noble products will not have negative effects on non-target organism in the soil if used at lower concentration level.

6. 5 References

Alengebawy, A., Abdelkhalek, S.T., Qureshi, S.R. and W.Q Wang. 2021. Heavy metals and pesticides toxicity in agricultural soil and plants: Ecological risks and human health implications. *Toxics* 9:1-33.

- Datta, S., Singh, J., Singh, S. and J. Singh. 2016. Earthworms, pesticides and sustainable agriculture: a review. *Environmental Science and Pollution Research* 23:8227-8243.
- Dionísio, J.A., Demetrio, W.C. and A. Maceda. 2018. Earthworms and Nematodes: *The Ecological and Functional Interactions*. IntechOpen.
- Jansirani, D., Nivethitha, S. and M.V.P Singh. 2012. Production and utilization of vermicast using organic wastes and its impact on *Trigonella foenum* and *Phaseolus aureus*. *International Journal of Research in Biological Sciences* 2:187-189.
- Jeyaprakasam, A., Muniyandi, B., James, A.J.P., Karmegam, N. and K. Ponnuchamy. 2021. Assessment of earthworm diversity and pesticide toxicity in *Eudrilus eugeniae*. *Environmental Chemistry and Ecotoxicology* 3:23-30.
- Maréchal, J., Hoeffner, K., Marié, X. and D. Cluzeau. 2021. Response of earthworm communities to soil engineering and soil isolation in urban landscapes. *Ecological engineering* 169:1-6.
- Mashela, P.W., De Waele, D., Dube, Z., Khosa, M.C., Pofu, K.M., Tefu, G., Daneel, M.S. and H. Fourie. 2017. Alternative Nematode Management Strategies. In: Fourie, H., Spaull, V.W., Jones, R.K., Daneel, M.S. and D. De Waele (eds.). *Nematology in South Africa: A View from the 21st Century*. Springer International Publishers: Heidelberg.
- Miglani, R. and S. S Bisht. 2019. World of earthworms with pesticides and insecticides. *Interdisciplinary toxicology* 12:71-82.
- Motulsky, H.J., 2007. Prism 5 statistics guide, 2007. GraphPad Software, 31:39-42.

- Muangphra, P. and R. Gooneratne. 2011. Toxicity of commercial neem extract to earthworms (*Pheretima peguana*). *Applied and Environmental Soil Science* 1:1-8.
- Ouina, T.S., Panoff, J.M., KoussÃ, M. and T.J Gonnety. 2017. Effect of pesticides and micro-organisms on earthworm *Eisenia fetida* (Savigny, 1826). *African Journal of Agricultural Research* 12:706-715.
- Pelosi, C., Lebrun, M., Beaumelle, L., Cheviron, N., Delarue, G. and S. Nelieu. 2016. Sublethal effects of epoxiconazole on the earthworm *Aporrectodea icterica*. *Environmental Science and Pollution Research* 23:3053-3061.
- Shahzad, A. and S.S. Pawar. 2017. Mortality (LC50) by standardization artificial soil test method for estimation of toxic effect of dieldrine and cythion on earthworm, *Eisenia fetida*. *International Journal of Innovative Research and Advanced Studies* 4:148-152.
- Yadav, J., Rani, M., Zhang, T.C. and U. Shanker. 2023. Efficient photo-adsorptive eradication of endocrine disrupting pesticides by chitosan co-decorated metal oxide bio-nanocomposite. *Environmental Science and Pollution Research* 30:72523-72538.
- Yatoo, A.M., Ali, M.N., Zaheen, Z., Baba, Z.A., Ali, S., Rasool, S., Sheikh, T.A., Sillanpää, M., Gupta, P.K., Hamid, B. and B. Hamid. 2022. Assessment of pesticide toxicity on earthworms using multiple biomarkers: a review. *Environmental Chemistry Letters* 20:2573-2596.

CHAPTER 7

SUMMARY, SIGNIFICANCE OF FINDINGS, FUTURE RESEARCH AND CONCLUSIONS

7.1 Summary

This study assessed a possible toxicity of Nemarioc-AL and Nemafric-BL phytonematicides on non-target organisms. Bioavailability of phytonematicides was determined on tomato roots, stems, leaves, fruits and different soil types. Then accumulation of cucurbitacin A and B residues were detected in smaller quantities of mg/kg. The cucurbitacin's are widely spread in our daily cuisines like in pumpkin and watermelon which should not be a challenge if found at such lower concentrations in produce treated by Nemarioc-AL and Nemafric-BL phytonematicides. I therefore, recommend the use of phytonematicides for plant protection purposes, because at a given concentration 3%, the accumulation of cucurbitacin residues was very low. However, when these products are used, care should be taken to avoid human exposure.

The CARD computer algorithm model was used to develop MCSP on tomato plants and overall sensitivity of the products. Using the biological indices, MCSP for Nemarioc-AL and Nemafric-BL phytonematicides on tomato plants inoculated by earthworms was 1.09 and 1.35%, respectively. The overall sensitivity of a crop to Nemarioc-AL and Nemafric-BL phytonematicides were $\sum k = 1$ unit and $\sum k = 2$ units, respectively. Demonstrating tomato plants were more sensitive to Nemarioc-AL phytonematicide than Nemafric-BL phytonematicide. Earthworms' variables were subjected to lines of the best fit. The increasing Nemarioc-AL phytonematicide

concentration exhibited a positive quadratic equation on earthworms' variables. In contrast, Nemafric-BL phytonematicide concentrations exhibited both positive and negative quadratic equation. Furthermore, earthworms were assessed at molecular level by proteomics by using one-dimensional analysis. At lower concentrations of Nemarioc-AL and Nemafric-BL phytonematicides protein bands were shallow and at higher concentration they intensified.

GraphPad prism was used to tackle the issue of cytotoxicity on Raw 264.7 macrophage cells and earthworms. Cytotoxicity of the phytonematicides was evaluated by using Raw 264.7 macrophage cells using microplate. Using MTT assay, the Nemarioc-AL and Nemafric-BL phytonematicides were toxic to Raw 264.7 macrophage at high concentrations and less toxic at lower concentrations. This was shown by increased cell viability at low concentration and reduced viability at high concentrations. The anti-inflammatory was assessed by using a microscope with both high and low concentrations showing signs of cytotoxicity on Raw 264.7 macrophage differing on their degree. Cell viability and mortality of earthworms were evaluated under *In vitro* conditions. The relationship of cell viability and mortality were inversely promotional to each other in the current study as affected by phytonematicide concentrations.

7.2 Significance of findings

The findings of this study have shown that the response of living entities to Nemarioc-AL and Nemafric-BL phytonematicides followed a density-dependent growth pattern (DDG). These DDG pattern is compartmentalized by stimulation, neutral and inhibition

phase. The stimulation phase, occurred at lower concentration levels by stimulating plant, earthworms' variables and Raw 264.7 macrophage suggesting non-toxic effects of phytonematicides. In contrast, inhibition occurred at high concentration levels resulting in mortality and structural deformity of earthworms. In this regard, low concentrations have indicated that phytonematicide are safe to be used for intended purpose. While signs of structural deformities at lower concentration on earthworms and Raw 264.7 macrophage explains a direct contact of this product can still be toxic at low degree.

7.3 Future research

Protein synthesis in response to phytonematicides brought crucial information on safety of the products on non-target organism. However, more studies need to be conducted based on molecular level including phytonematicide effects on earthworm DNA profile. The phytonematicides contain secondary metabolites which are allelochemical that can either stimulate or inhibit other species. There is still a dearth of information on the effects of phytonematicides on un-targeted metabolites. Since the product has plethora of phytochemicals cucurbitacin B which is mostly renowned as an anticancer property. A study should be conducted to evaluate its effects on cancer.

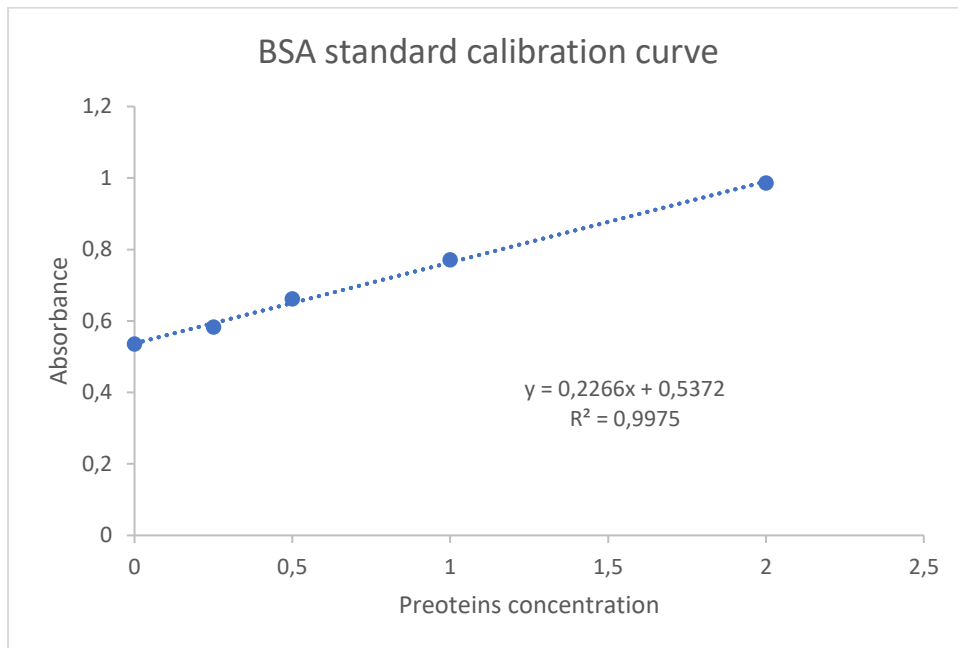
7.4 Conclusion

The Nemarioc-AL and Nemafric-BL phytonematicides has shown a moderate toxicity to living entities at low concentration level. As a product developed to manage plant parasitic nematodes moderate toxicity of a product is not an issue. While high

concentration levels should be avoided as this product has shown acute toxicity at that degree. Therefore, these products are viewed as a future alternative bionematicide which is cost effective and environmentally friendly.

APPENDICES

Appendix 1. The figure shows the standard curve of Protein concentration vs Absorbance used to estimate protein concentration from samples.



Appendix 2. Standard calibration curve of pure cucurbitacin A used to estimate cucurbitacin A accumulation in a sample.

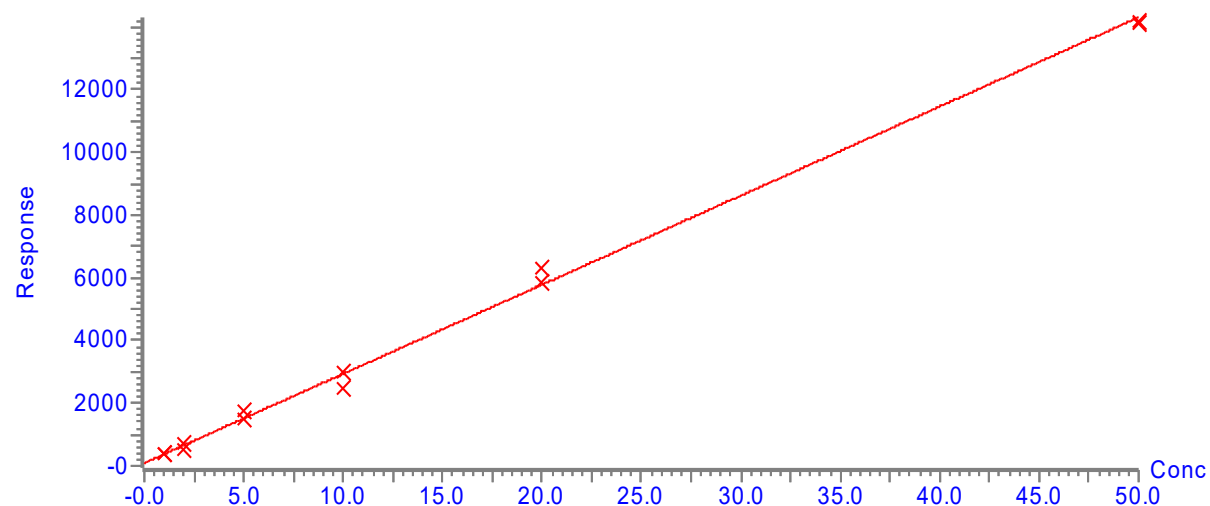
Compound name: Cucurbitacin A

Correlation coefficient: $r = 0.997100$, $r^2 = 0.994209$

Calibration curve: $284.325 * x + 91.5253$

Response type: External Std, Area

Curve type: Linear, Origin: Exclude, Weighting: 1/x, Axis trans: None



Appendices 3. Standard calibration curve of pure cucurbitacin A used to estimate cucurbitacin B accumulation in a sample.

Compound name: Cucurbitacin B

Correlation coefficient: $r = 0.996778$, $r^2 = 0.993566$

Calibration curve: $398.219 * x + -35.7933$

Response type: External Std, Area

Curve type: Linear, Origin: Exclude, Weighting: 1/x, Axis trans: None

