CUCUMIS MYRIOCARPUS BIOTEST SOLUTIONS WITH ANTIHELMINTIC AND ANTIBACTERIAL PROPERTIES

BY

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DECLARATION

I hereby declare that the information recorded herein was originated and compiled by myself and that it was never presented elsewhere. Due acknowledgement was paid to the authors whose work had been cited.

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ABSTRACT

Experiments were conducted to select suitable solvents for extracting nematicidal and bacterial chemical compounds from ground fruits of wild cucumber (*Cucumis myriocarpus*). Solvents and mixtures tested include 80% hexane: 20% dichloromethane, 80% n-hexane: 20% methanol, 20% n-hexane: 80% methanol, 100% dichloromethane, 80% n-hexane: ethanol, 100% methanol, 100% acetone, 100% toluene, 100% water, 100% ethyl acetate, 100% petroleum ether, 100% n-hexane, total ethanolic extract, 100% hexane fraction, hexane-ethyl acetate (1:1, v/v) fraction, 100% ethyl acetate fraction and 100% methanol fraction. Selection of the solution with the highest antihelmtic effect was done through the standard bioactivity tests.

The nematode species used in the bioactivity tests were the root-knot nematode (*Meloidogyne incognita*) and the citrus nematode (*Tylenchulus semipenetrans*). In *M. incognita* study, the bioactivity effect ranged from 87% to 95%, whereas in *T. semipenetrans* the range was from 83% to 96%. The ethanolic extract and 100% hexane fraction were the best solvents for use in assessing antihelmintic properties of *C. myriocarpus* fruit.

The solvents used in antihelmintic studies, as well as 100% dichloromethane, 100% water, 100% acetone, 100% toluene, 100% petroleum ether and 100% n-hexane were tested for antibacterial properties. The bacteria used were a mixture of *Bacillus* species, namely, *B. litcheniformis*, *B. laterosporus* and *B. chitinosporus*. Extracts from *C. myriocarpus* fruit exhibited antibacterial properties towards *Bacillus* species. In all tests,

100% dichloromethane and 100% n-hexane were the best solvents for use in assessing antibacterial properties of *C. myriocarpus* fruits.

Minimum inhibitory concentration (MIC) of the solvents were determined using the biotest solutions extracted from 70.1 g *C. myriocarpus*. The 100% ethyl acetate and 100% petroleum ether had the lowest MIC of 3.13 mg/ml each, whereas in the 500 g material, hexane-ethyl acetate (1:1, v/v) and 100% ethyl acetate had the MIC of 0.78 mg/ml each.

Results of the study confirmed the antihelmintic properties of *C. myriocarpus* fruits. Also, they demonstrated for the first time the antibacterial properties of *C. myriocarpus* fruits towards *Bacillus* species.

CHAPTER 1

INTRODUCTION

Certain plant organs contain biologically active compounds, some of which have antimicrobial properties (Mitscher, Drake, Golloapudi and Okwute, 1987). Plant-derived chemicals are gaining attention in modern agriculture due to their environmental-friendliness (Ballesteros, Martin and Uriz, 1992). Considering the negative and often-cited incidences of synthetic chemicals against non-target organisms, there is a constantly rising need for new and environment-friendly bio-pesticides (Arnold and McLachlan, 1996; Krol, Arsenault, Pylypiw and Mattina, 2000; Mitscher *et al.*, 1987). Following the suspension of most halogenated pesticides, several plant species were tested for bioactivity on microbes and plant pests (Arnold and McLachlan, 1996; Krol *et al.*, 2000; Mitscher *et al.*, 1987).

Plant compounds that have potential pesticide properties have been high on the list of alternatives to synthetic pesticides (Ballesteros *et al.*, 1992). One prominent advantage of the use of biopesticides is their environment-friendliness, a criterion that demerits pesticides (Ballesteros *et al.*, 1992). Certain essential microbes, such as effective microbes, are of paramount importance as biological pesticides and for the decomposition of soil organic matter (Glare and O'Callaghan, 2000).

Crude extracts of wild cucumber (*Cucumis myriocarpus*) fruits were shown to have antihelmintic properties, through the ground leaching technology (GLT) under field, microplot and greenhouse conditions (Mabitsela, 2005; Mashela, 2002; Mashela and Mphosi, 2001; Mphosi, 2004). Extracts of *C. myriocarpus* fruit increased the efficacy of nitrogen-fixing bacteria (*Rhizobium*) under greenhouse (Mashela and Muedi, 2003)

and microplot (personal communication: Prof. Mashela) conditions. However, under field conditions *Bacillus* species did not interact with ground *C. myriocarpus* fruits (Mabitsela, 2005; Mphosi, 2004), castor bean (*Ricinus communis*) fruits (Mabitsela, 2005) and fever tea (*Lippia javanica*) leaves (Mabitsela, 2005; Ngobeni, 2003).

Ground *C. myriocarpus* fruits reduced nematode numbers, improved tomato and cowpea productivity and increased soil electrical conductivity, but had no effect on soil pH (Mashela, 2002; Mphosi, 2004; personal communication: Prof. Mashela). Khosa (2005) demonstrated that the efficacy of *C. myriocarpus* on nematode suppression was comparable with that of aldicarb and phenamiphos. Mofokeng (2005) demonstrated that the *C. myriocarpus* was non-host to *M. incognita*.

Bioactivity tests are used to evaluate the influence of plant extracts on the activities of organisms under laboratory conditions. The tests are used for both screening of the materials and for assessing the minimum inhibitory concentration (MIC) of the test solutions on target organisms. In this study, the bioactivity tests were used to identify solvents that extract chemical compounds in *C. myriocarpus* fruit which have both antihelmintic and antibacterial properties. The specific objectives of the study were:

(i) To determine the antihelmintic properties of *C. myriocarpus* fruit extracts using various solvents against the root-knot nematode (*Meloidogyne incognita*) and the citrus nematode (*Tylenchulus semipenetrans*).

(ii) To determine the antibacterial activities of *C. myriocarpus* fruit extracts using various solvents against *Bacillus* species.

(iii) To determine the MIC of *C. myriocarpus* fruit extracts using various solvents against *Bacillus* species.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

A large number of plant species are being assessed for their bioactivity against plant pests (Arnold and McLachlan, 1996; Krol *et al.*, 2000; Mitscher *et al.*, 1987). Bioactivity tests are used to assess the efficacy of various extracts from plants on pests. Also, the tests could be used to identify an appropriate solvent for a particular plant organ with known bioactivity. Ground wild cucumber (*Cucumis myriocarpus*) fruits were shown to have antihelmintic properties under a wide range of conditions (Khosa, 2005; Mabitsela, 2005; Mashela, 2002; Mashela and Mphosi, 2001; Mofokeng, 2005; Mphosi, 2004). Also, the absence of interactions between *C. myriocarpus* and *Bacillus* species suggested that *C. myriocarpus* might be poisonous to *Bacillus* species (Mabitsela, 2005; Mphosi, 2004). The ensuing literature review is intended to evaluate the work that had been done in *C. myriocarpus* in relation to nematode suppression and bioactivity tests of various plants using nematodes and bacteria as target organisms.

2.2 *Cucumis myriocarpus*

Ground *C. myriocarpus* fruits suppressed nematode egg-hatch in laboratory conditions by 97-99%, whereas under greenhouse conditions *M. incognita* juveniles numbers were reduced by 92-93% in soil (Mashela, 2002). Under both conditions *C. myriocarpus* crude fruit extract increased electrical conductivity (EC), but had no effect on soil pH. The release of toxic compounds from *C. myriocarpus* fruit extracts is believed to be independent of soil microorganisms, suggesting that the toxic compounds are water-soluble (Mashela, 2002).

Under field studies, *C. myriocarpus* crude fruit extract was independent of the activities of *Bacillus* species (Mabitsela, 2005; Mphosi, 2004), confirming the ground leaching technology (GLT) which suggests that microbial decomposition was not a prerequisite for the nematicidal activity (Mashela, 2002). Briefly, the technology involves using small quantities of toxic organs in powdered form to suppress plant-parasitic nematodes. Mashela (2002) suggested that microbial decomposition was not essential for the release of nematicidal compounds in this technology, and that the compounds were leached out of organic matter through irrigation water. *Cucumis myriocarpus* crude fruit extract increased tomato fruit yield, stem diameter, plant weight and soil EC by 61%, 99%, 74% and 68%, respectively (Mphosi, 2004). Also, cowpea inoculated with *Bradyrhizobium* had higher nodule number and weight under soil amended with ground *C. myriocarpus* fruits in greenhouse (Mashela and Muedi, 2003) and field (personal communication: Prof Mashela) studies.

Mashela (2002) demonstrated that densities of *M. incognita* in soil and roots were reduced by 49% and 83%, respectively, in spring, whereas soil and root nematodes decreased by 68% and 73%, respectively, in autumn studies. Tomato plant weight, plant height and fruit weight also increased. In another field study, the efficacy of *C. myriocarpus* crude fruit extract was comparable to that of aldicarb and phenamiphos on nematode suppression and improvement in tomato productivity (Khosa, 2005).

Toxic components in *C. myriocarpus* crude are cucumis ($C_{27}H_{40}O_9$) and leptoderms ($C_{27}H_{38}O_8$), collectively referred to as cucurbitacins (Van Wyk, Van Oudtshoorn and Gericke, 1997). A separate study in our group aimed at extraction and identification of the toxic components is currently underway, and it is hoped that the results will shed

more light on the structure-activity relationship. Cucurbitacins accumulate in fruits and in roots, but not in leaves. Under microplot conditions, *C. myriocarpus* roots did not support the reproduction of the root-knot nematode (Mofokeng, 2005). The water-soluble cucurbitacins are amongst the bitterest substances known to man (Jeffery, 1978; Rimington, 1998). Although *C. myriocarpus* fruits and roots are widely used by traditional healers for various ailments, almost always, overdoses result into fatalities (Duke, 1992a; Rimington, 1998).

2.3 Bioactivity against nematodes

Either fresh or air-dried plant materials are used in bioactivity tests (Mojumder, Mishra, Haque and Goswami, 1989; Naqvi, Khan, Shaikh and Shaikh, 1992; Sundararaju, Banu and Ratnakaran, 1994). The most commonly used extract solvents include water (Khurma and Mangotra, 1999; Naqvi *et al.*, 1992; Sundararaju *et al.*, 1994), methanol (Alen, Nakajima, Nitoda, Baba, Kanzaki and Kawazu, 2000; Mackeen, Ali, Abdullah, Nasir, Mat, Razak and Kawazu, 1997), acetone (Sundararaju *et al.*, 1994) and ethanol (Naqvi *et al.*, 1992). Once the material is extracted from plant tissues, it is separated from plant debris using Whatmann paper no. 1. The solvent is separated from the filtrate through evaporation, usually at 40-45^{o}C (Lall and Meyer, 2000; Naqvi *et al.*, 1992; Rabanal, Arias, Prado, Hernandez-Perez and Sanchez-Mateo, 2002). Prior to use, the concentrated materials are stored at 4^{o}C (Lall and Meyer, 2000; Rojas *et al.*, 2003; Sokmen *et al.*, 1998).

Nematode juveniles are extracted using the modified Baermann method (Rodriguez-Kabana and Pope, 1981). The advantage of this method is that only live second stage juveniles (J2s) are extracted. Generally, the J2s are immediately used to ensure that

fresh nematodes are subjected to the tested chemical compounds. Both free-living and plant-parasitic nematodes had been used in bioactivity studies. The most widely used free-living nematode is *Caenorhabditis elegans* (Halbrendt and Jing, 1994), whereas plant-parasitic nematodes include *Bursaphelenchus xylophilus*, *M. incognita*, *Hoplolaimus indicus* and *Radopholus similis* (Alen *et al.*, 2000; Khurma and Mangotra, 1999; Mackeen *et al.*, 1997; Mojumder *et al.*, 1989; Sundararaju *et al.*, 1994).

The concentrated plant materials are diluted either in distilled water (Qamaruddin, Parveen, Khan and Singhal, 2002) or DMSO (Alzoreky and Nakahara, 2003; Rabanal, Arias, Prado, Hernandez-Perez and Sanchez-Mateo, 2002) to form biotest solutions. Dilutions could either be serial or non-serial (Rabanal *et al.*, 2002). Once nematodes are subjected to the biotest solutions, they are incubated at approximately 27 ^{o}C (Khurma and Mangotra, 1999). Nematodes are counted at various intervals, usually varying from 12 to 72 hours (Halbrendt and Jing, 1994; Haseeb, Singh, Khan, and Saxena, 1978; Sundararaju *et al.*, 1994).

2.4 Bioactivity against bacteria

A large number of bacterial species had been subjected to bioactivity tests of plant materials. Some of the used bacteria species include: *Bacillus subtilis*, *B. cereus*, *B. coagulans*, *B. megaterium*, *Listeria mononcytogenes*, *Staphylococcus aureus* (Alzoreky and Nakahara, 2002; Enzo, Palombo and Semple, 2001; Essawi and Srour, 2000; Khan and Omoloso, 2003; Khan, Omoloso and Kihara, 2003; Kone, Antindehou, Terreaux, Hostettmann, Traore and Dosso, 2004; Negi, Anandhamakrishnan and Jayaprakasha, 2003; Pinheiro, Nakamura, Filho, Ferreira, Young and Gomez, 2003).

The solvents used include: dichloromethane, methanol, ethanol, acetone, n-hexane, water, ethyl acetate and toluene (Alzoreky and Nakamura, 2002; Hernandez, Canales, Avila, Duran, Caballero, de Vivar and Lira, 2003; Khan *et al.*, 2003; Machado *et al.*, 2002; Negi *et al.*, 2003; Neto, Owens, Langfield, Comeau, Onge, Vaisberg and Hammond, 2002; Nostro, Germano, D'Angelo, Marino and Cannatelli, 2000; Okoli and Iroegbu, 2004; Pessini, Filho, Nakamura and Cortez, 2003; Truiti, Sarragiotto, Filho, Nakamura and Filho, 2003). The listed solvents extract various chemical compounds from plant tissues, with most of them extracting common chemical compounds (Appendix 1).

Generally, the procedure for preparing the biotest solution for bacterial bioactivity tests is similar to that expounded in helmintic bioactivity tests (section 2.3). However, the major difference is that a growth-promoting medium is also prepared for the culturing of bacteria. Bacteria are cultured in nutrient broth (Bassole, Ouattara, Nebie, Ouattara, Kabore and Traore, 2003; Gaidamashvili and Van Staden, 2001; Nascimento, Locatelli, Freitas and Silva, 2000; Truiti *et al.*, 2003). The most commonly used growth medium in trials is the Mueller-Hinton agar (Bonjar and Nik, 2004; Palombo and Semple, 2001).

2.4.1 Bioactivity methodologies

The plate-hole diffusion assay is used to determine the inhibition of bacterial growth by plant extracts (Palombo and Semple, 2001). An amount of 200 µl of 24-hour-old nutrient broth culture is added into 15-ml of molten Mueller-Hinton agar, mixed, poured into a sterile Petri dish and allowed to set. A sterile cork-borer (5-mm

diameter) is used to make wells in the set agar. Approximately 25 µl of plant extract, with 1:200 plant: water (v/v) dilutions, are added to each well and the plates are incubated at 37°C for 24 hours. Antibacterial activity is recorded by measuring the diameter of a circular bacterial growth from the centre of the 5-mm well towards the perimeter of the Petri dish.

The estimate of the MIC is carried out by the broth microdilution method in microplates (Ellof, 1998; Rabanal *et al.*, 2002; Rhajaoui, Oumzil, Faid, Lyagoubi, Elyachioui and Benjouad, 2001). From an initial extract, dilutions of various concentrations are prepared and buffered to pH 7. Five µl of bacterial suspension contains 10^5 bacteria per µl (Rhajaoui *et al.*, 2001). The bacteria-extract mixture is incubated at 37°C for 24 hours. Bacterial growth is assessed by adding p-iodonitrotetrazolium violet solution into microplates and observing colour change (Reiner, 1982). The first colour change represents the MIC for the biotest solution being evaluated.

CHAPTER 3

CUCUMIS MYRIOCARPUS BIOTEST SOLUTIONS WITH ANTIHELMINTIC PROPERTIES

3.1 Introduction

Bioactivity test is used to assess the impact of chemicals on living organisms (Hench and Wilson, 1993). Most of the bioactivity tests on nematodes were conducted using the free-living nematodes (Momin and Nair, 2002; Sparg, Van Staden and Jager, 2001). Free-living nematodes feed on bacteria, fungi or other nematodes, and they are generally active. Ground wild cucumber (*Cucumis myriocarpus*) fruits reduced densities of the root-knot (*Meloidogyne incognita*) nematode under various conditions (Mabitsela, 2005; Mashela, 2002; Mphosi, 2004). The objective of this study was to select the solvents that extract chemical compounds in *C. myriocarpus* fruits which have antihelmintic properties on plant-parasitic nematodes.

3.2 Materials and Methods

The experiment was initiated on 18 October 2004 in the VLIR Nematology Laboratory, University of Limpopo. Fruits of *C. myriocarpus* were locally collected, dried for 5 days in air-forced oven at 52^{o}C to minimize the loss of volatile phytochemicals and ground in a Wiley mill to pass through a 1-mm sieve. Powdered fruit material (500 g) was extracted with ethanol for 24 hours at room temperature. The ethanol extract was filtered using Whatmann filter paper no. 1 and the filtrate was evaporated using a rotavapor at 50^{o}C. Liquid-liquid fractionation was done from the ethanol extract, using hexane, (1:1, v/v) hexane-ethyl acetate, ethyl acetate and methanol. The remaining aqueous extract was retained as ethanolic extract. Fractions and ethanolic extract were evaporated at 50^{o}C to dryness. Prior to the bioassay, each

evaporated material was resuspended in Dimethylsulphoxide (DMSO), concentrated to 100 mg/ml and stored at 4°C (Lall and Meyer, 2000; Rojas *et al.*, 2003; Sokmen, Jones and Erturk, 1998). The test solutions were water (B_1), DMSO (B_2), total ethanolic extract (P_1), 100% hexane fraction (P_2), hexane-ethyl acetate (1:1, v/v) fraction (P_3), 100% ethyl acetate fraction (P_4) and 100% methanol fraction (P_5), where B_1 and B_2 served as untreated test solutions.

***Meloidogyne incognita*:** Second stage juveniles (J2s) of *M. incognita* were collected from roots of Swiss Chard (*Beta vulgaris*) growing under field conditions. Roots were placed into a plastic bag, 1:10 sodium hypochlorite: water (v/v) solution added, and mechanically shaken on Labcon shaking machine for 5 minutes at 75 rounds per minute (rpm) to dislodge juveniles and eggs from roots. Juveniles and eggs were separated from debris by passing through a series of sieves: 150 µm, 75 µm, 63 µm, 45 µm and 25 µm pore sieves. Juveniles and eggs were collected from the 25 µm pore sieves into 500-ml plastic beakers. Kleenex paper was placed on a 250 µm-pore sieve in 20-cm diameter plastic dish and contents of the 500-ml beakers were added on the paper to extract second stage juveniles (J2s) for a period of 3 days. The aliquot was concentrated on a 25 µm pore sieve and the J2s were washed into a 500-ml measuring cylinder and tapwater added to a 280-ml mark.

Approximately 10-ml aliquot containing J2s was pipetted into 120-ml plastic nematode bottles with caps. The B_1 and B_2 non-biotest solutions and P_1, P_2, P_3, P_4 and P_5 biotest solutions were each added into nematode-containing bottles using a precision pipette with a total volume of 200 µl. Labelled bottles were closed and mechanically shaken for 5 minutes at 75 rpm in order to mix the biotest solutions with aliquots. The seven

treatments, B_1, B_2, P_1, P_2, P_3, P_4 and P_5 were placed in the laboratory shelf at 25 °C in a completely randomized design (CRD), with five replications.

Live and dead nematodes were counted from a 10-ml aliquot under a light microscope on day 2, 4, 6 and 8. During each count, the counted nematodes were returned to the plastic bottle, closed, hand-shaken and placed in the shelf. Prior to analysis of variance (ANOVA) data were transformed using a logarithm method, Ln (1 + x), in order to homogenize the variance. However, untransformed data were reported. When the treatment means were different ($P \leq 0.05$), mean separation was done using the least significant difference test (Gomez and Gomez, 1984). The effect of exposure time per biotest solution was also evaluated. Means were reported using bar diagrams (Figures 3.1 – 3.11).

Tylenchulus semipenetrans: The study was initiated on 4 November 2004. *Tylenchulus semipenetrans* juveniles and eggs were collected from Zebediela Citrus Estate, Limpopo Province, and extracted as described for *M. incognita*. The aliquot was concentrated to 500-ml, after a 3-day incubation period, juveniles were pipetted into 120-ml bottles using a 10-ml syringe. The seven treatment solutions were prepared as described earlier. Treated aliquots were arranged in a CRD, with six replications.

The aliquots were mechanically mixed with the biotest solutions through shaking for 5 minutes at 75 rpm. Because of excessively high nematode counts, the nematode solutions in the bottles were diluted with 100-ml tapwater 8 days after initiating the treatments. Dead and live nematodes were counted from a 10-ml aliquot under a light

microscope. The numbers of dead nematodes obtained from the 10-ml aliquot were converted to the original undiluted 10-ml to obtain the number of nematodes per container. The study was repeated on 15 November 2004. Data analysis was as described for *M. incognita* study.

3.3 Results

The bioassay mortality trends suggested that biotest solutions increased the mortality of nematode juveniles. Mortality trends became much more obvious from the fourth through the eighth day. Similarly, in terms of exposure time, bioassay mortality trends suggested that nematode mortality did not differ over time.

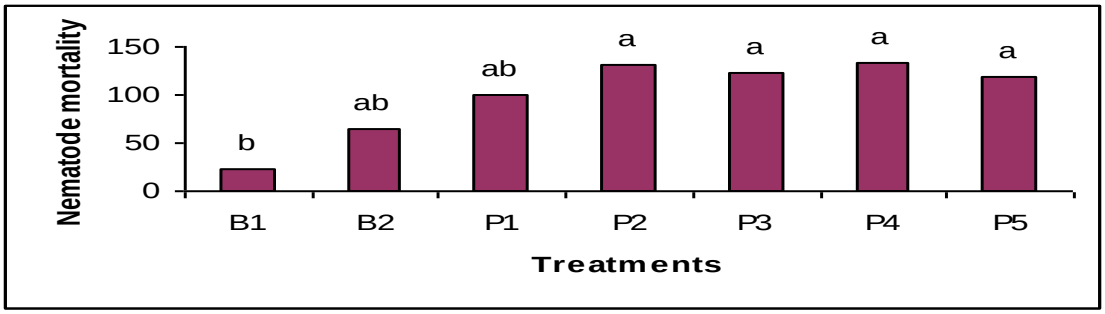
3.3.1 *Meloidogyne incognita*

The data provided two separate sets of information: (i) comparison of mean effect of the seven biotest solutions on nematode mortality, (ii) mean nematode mortality of each biotest solution over a period of eight days.

(a) Comparisons of biotest solutions

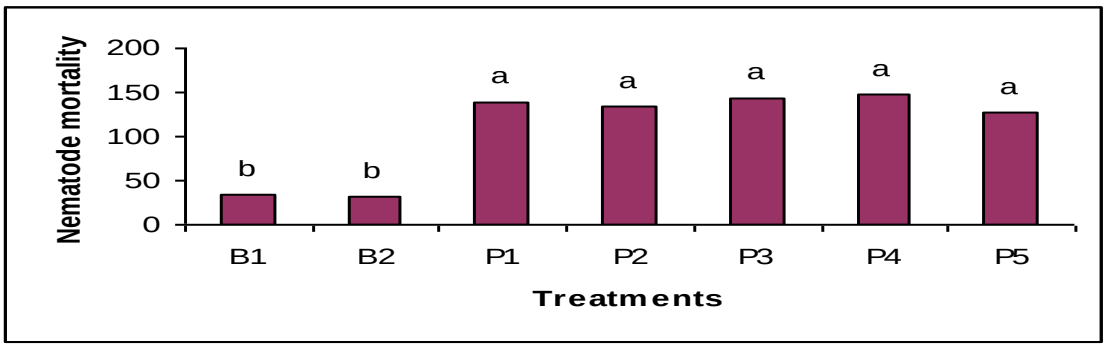
Day two: Two days after initiating the treatments, the bioactivity of the fractions on nematode mortality did not differ from those of the P_1 biotest solution and the B_2 non-biotest solution. Although the effect of water on nematode mortality did not differ from those of DMSO and ethanolic extract, it differed from those of other biotest solutions (Figure 3.1).

Day four: Four days after initiating the treatments, effects of the extract (P_1) and all fractions (P_{2-5}) on nematode mortality did not differ (Figure 3.2). However, the effects of the five biotest solutions differed from those of the non-biotest solutions.



B_1 = water; B_2 = DMSO; P_1 = total ethanolic extract; P_2 = 100% hexane fraction; P_3 = hexane-ethyl acetate (1:1, v/v) fraction; P_4 = 100% ethyl acetate fraction; P_5 = 100% methanol fraction.

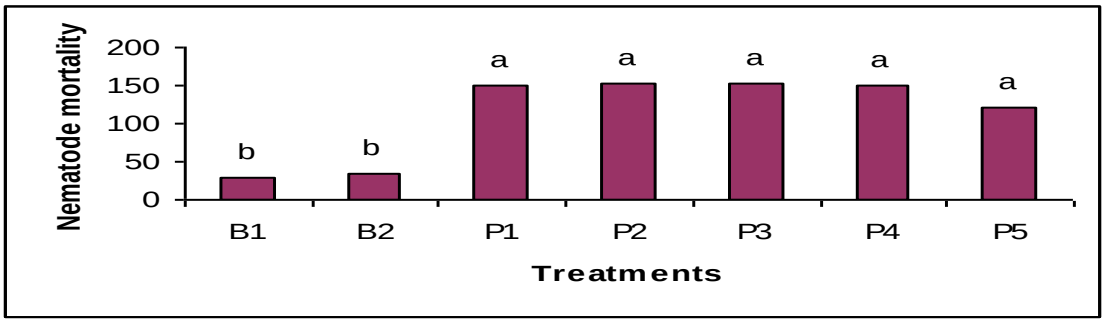
Figure 3.1 Effects of biotest solutions of *Cucumis myriocarpus* fruits on *Meloidogyne incognita* over two days



B_1 = water; B_2 = DMSO; P_1 = total ethanolic extract; P_2 = 100% hexane fraction; P_3 = hexane-ethyl acetate (1:1, v/v) fraction; P_4 = 100% ethyl acetate fraction; P_5 = 100% methanol fraction.

Figure 3.2 Effects of biotest solutions of *Cucumis myriocarpus* fruits on *Meloidogyne incognita* over four days

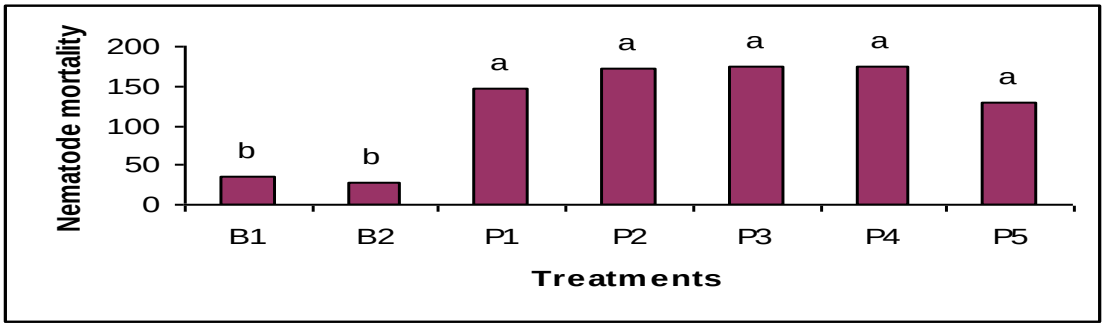
Day six: Six days after initiating the treatments, effects of biotest solutions on nematode mortality did not differ (Figure 3.3). However, when compared with the non-biotest solutions, the materials resulted in higher nematode mortalities. The impact of the two non-biotest solutions on nematode mortality did not differ.



B_1 = water; B_2 = DMSO; P_1 = total ethanolic extract; P_2 = 100% hexane fraction; P_3 = hexane-ethyl acetate (1:1, v/v) fraction; P_4 = 100% ethyl acetate fraction; P_5 = 100% methanol fraction.

Figure 3.3 Effects of biotest solutions of *Cucumis myriocarpus* fruits on *Meloidogyne incognita* over six days

Day eight: Eight days after initiating the treatments, effects of biotest solutions on nematode mortality did not differ (Figure 3.4). When compared with the two non-biotest solutions, the biotest solutions increased nematode mortality. However, the effect of the two non-biotest solutions did not differ from each other.



B_1 = water; B_2 = DMSO; P_1 = total ethanolic extract; P_2 = 100% hexane fraction; P_3 = hexane-ethyl acetate (1:1, v/v) fraction; P_4 = 100% ethyl acetate fraction; P_5 = 100% methanol fraction.

Figure 3.4 Effects of biotest solutions of *Cucumis myriocarpus* fruits on *Meloidogyne incognita* over eight days

(b) Exposure time

When nematodes were exposed to total ethanolic extract (P_1), the exposure time had no effect on the mortality of nematodes during an eight day period (Figure 3.5).

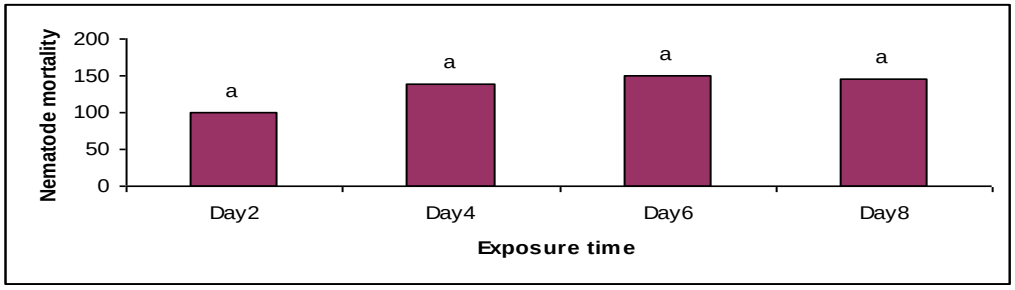


Figure 3.5 Effects of total ethanolic extract (P_1) from *Cucumis myriocarpus* fruits on mortality of *Meloidogyne incognita* over an exposure period of eight days

Nematode mortality when exposed to 100% hexane fraction (P_2) was dependant on the exposure duration. More nematodes were dead on day 8 compared to day 2 and day 4 (Figure 3.6). However, during day 6 nematode mortality did not differ from those on days 2, 4 and 8.

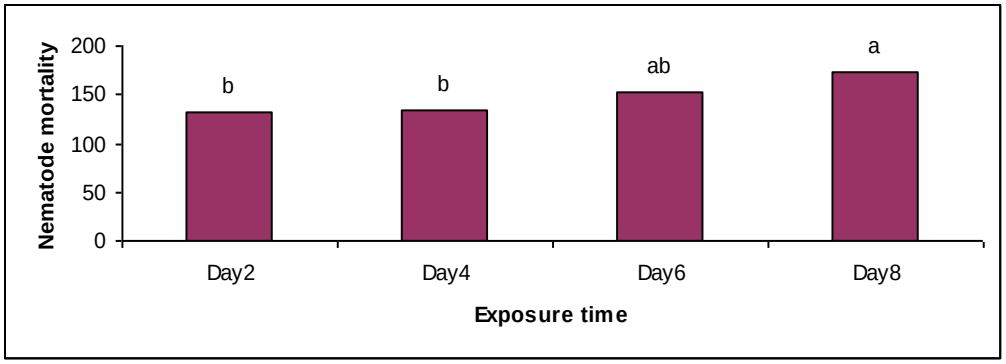


Figure 3.6 Effects of 100% hexane fraction (P_2) from *Cucumis myriocarpus* fruits on mortality of *Meloidogyne incognita* over an exposure period of eight days

Nematodes mortality when exposed to hexane: ethyl acetate (1:1 v/v) fraction (P_3) was also dependent on the exposure time. Most nematode had died on day 8 compared with day 2, whereas nematode mortality in day 2 did not differ from those in days 4 and 6 (Figure 3.7). Similarly, mortality in day 8 did not differ from those in days 4 and 6.

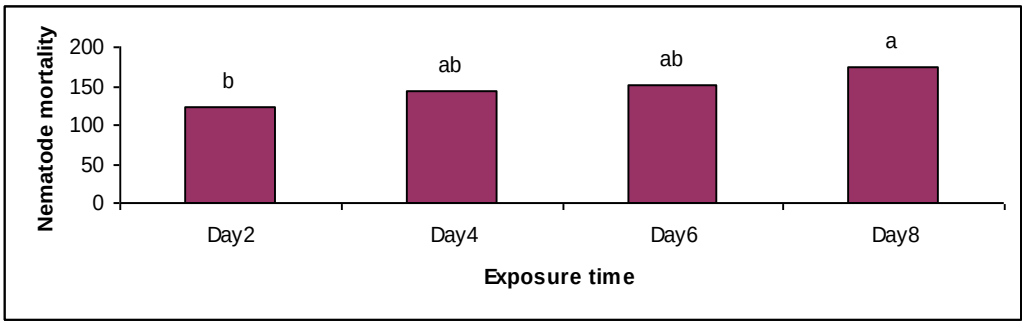


Figure 3.7 Effects of hexane: ethyl acetate (1:1, v/v) fraction (P_3) from *Cucumis myriocarpus* fruits on mortality of *Meloidogyne incognita* over an exposure period of eight days

In 100% ethyl acetate fraction (P_4), nematode mortality was also not affected by exposure time (Figure 3.8). In 100% methanol fraction (P_5), nematode mortality was also not affected by exposure time (Figure 3.9).

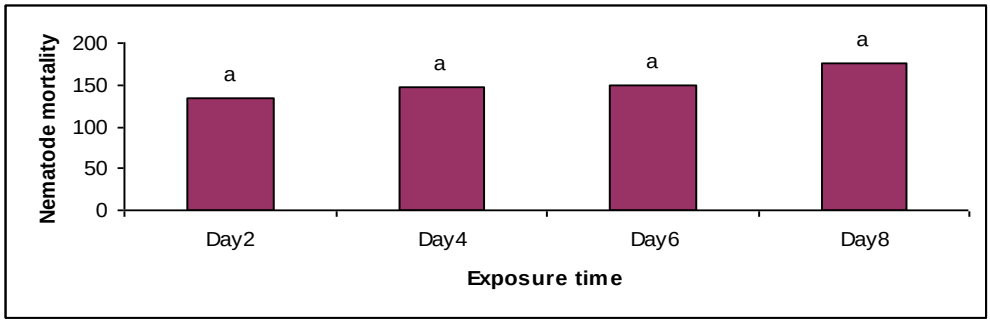


Figure 3.8 Effects of 100% ethyl acetate fraction (P_4) from *Cucumis myriocarpus* fruits on mortality of *Meloidogyne incognita* over an exposure period of eight days

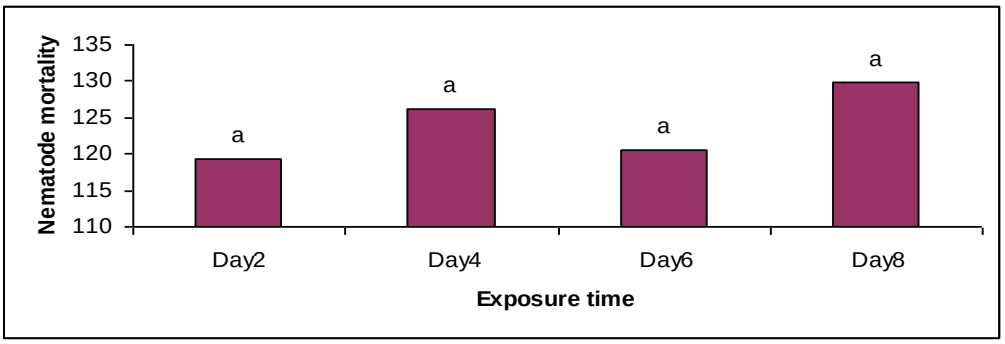


Figure 3.9 Effects of 100% methanol fraction (P_5) from *Cucumis myriocarpus* fruits on mortality of *Meloidogyne incognita* over an exposure period of eight days

Nematode mortality in water (B_1) was fairly low and approximately constant from day 2 to day 8, suggesting that the death of nematodes was natural (Figure 3.10).

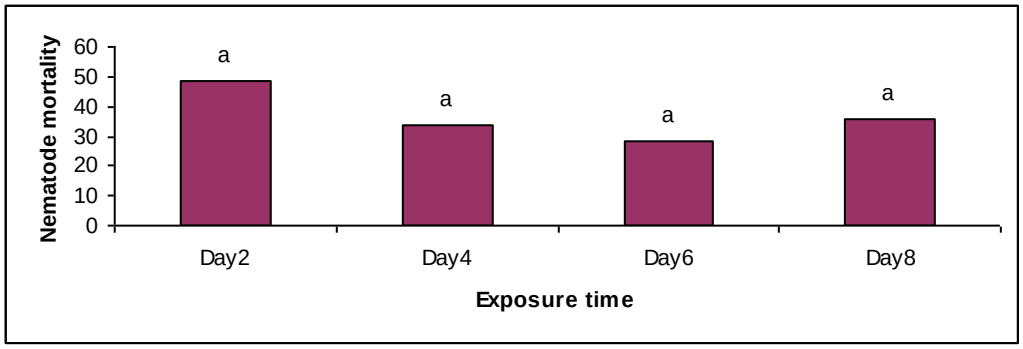


Figure 3.10 Effects of water (B_1) on mortality of *Meloidogyne incognita* over an exposure period of eight days

Nematode mortality in DMSO (B_2) was fairly low and approximately constant from day 2 to day 8, suggesting that nematodes were dying due to natural causes (Figure 3.11).

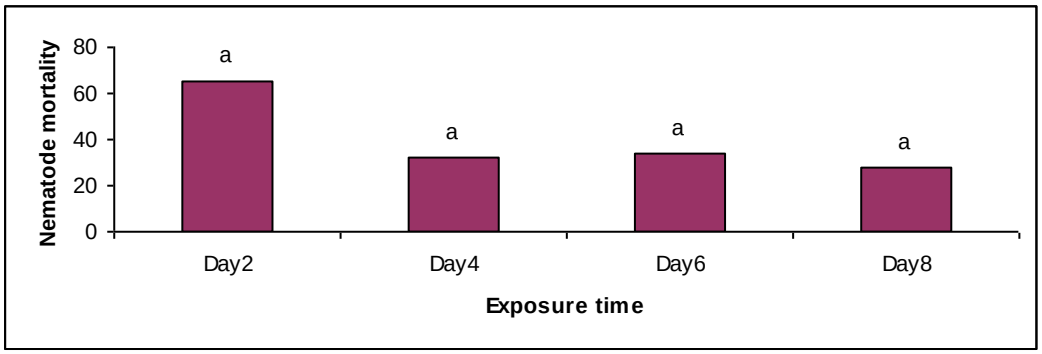
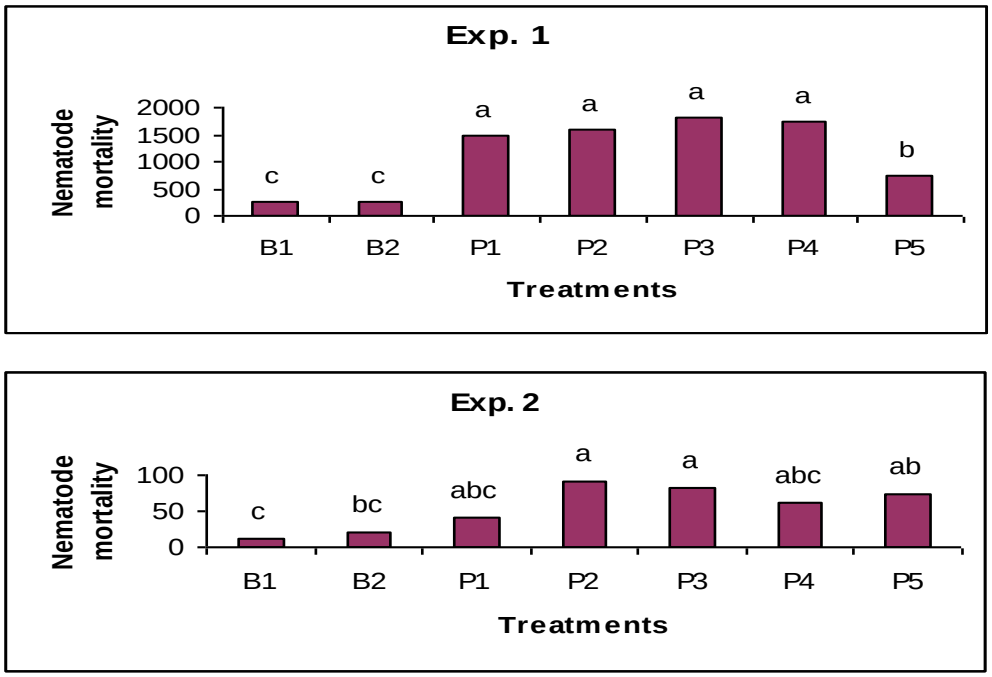


Figure 3.11 Effects of DMSO (B_2) on mortality of *Meloidogyne incognita* over an exposure period of eight days

3.3.2 *Tylenchulus semipenetrans*

Eight days after initiating the treatments, the impact of biotest solutions on nematode mortality was higher than of non-biotest solutions in Experiment 1 (Figure 3.12). In Experiment 2, the data were variable, with distinct differences on nematode mortality being observed under two biotest solutions (P_2, P_3) and non-biotest solutions.

However, the effects of P_2 and P_3 did not differ from those of P_1, P_4 and P_5. Nematode mortality in non-biotest solutions did not differ from each other or those in P_1 and P_4, although that in B_1 was significantly lower than that in B_2.



1 = water (B_1); 2 = DMSO (B_2); 3 = total ethanolic extract (P_1); 4 = 100% hexane fraction (P_2); 5 = hexane-ethyl acetate (1:1, v/v) fraction (P_3); 6 = 100% ethyl acetate fraction (P_4); 7 = 100% methanol fraction (P_5).

Figure 3.12 Effects of biotest solutions of *Cucumis myriocarpus* fruits on *Tylenchulus semipenetrans* over eight days.

3.4 DISCUSSION

Biotest solutions from *C. myriocarpus* fruit extracts demonstrated that *C. myriocarpus* fruits have nematicidal properties. The various chemical solvents used in this study have the ability to extract various chemical compounds from plant tissues. Appendix 1 demonstrates that there are common chemical compounds across the listed chemical solvents used in this study. Various chemical compounds from a single plant material confer synergistic nematicidal properties (Kirkegaard and Agnus, 1996), resulting in high nematode mortality.

Solvents used in this study confirm results in other studies where the solvents extracted nematicidal compounds (Alen *et al.*, 2000; Mackeen *et al.*, 1997; Naqvi *et al.*, 1992; Sundararaju *et al.*, 1994). Biotest solution from ethanolic extracts of *Scilla natalensis*

demonstrated potent bioactivity against *Caenorhabditis elegans*, where 50% nematode mortality was recorded 2 hours after initiating the treatment at 25°C (Sparg *et al.*, 2001). Also, biotest solution from *Daucus carota* seeds, extracted using hexane, had antihelmintic effects on *C. elegans* and *Panagrellus redivivus* (Momin and Naire, 2002). Methanolic extracts of *Bruca sumatrana* and *Hoya diversifolia* also demonstrated nematicidal effects on *Bursaphelenchus xylophilus* (Alen *et al.*, 2000).

Generally, the cited bioactivity tests were conducted on free-living nematodes. In studies where plant-parasitic nematodes were used, the plant materials tested also demonstrated nematicidal effects (Ferris, Castro, Caswell, Jaffee, Roberts, Westerdahl and Williamson, 1992; Khurma and Mangotra, 1999; Zareen, Zaki and Javed, 2003). The explanation for the nematicidal impact on plant-parasitic nematodes was in terms of active compounds such as polythienyls, isothiocyanates, glucosinolates, cyanogenic glycosides, polyacetylenes, alkaloids, lipids, terpenoids, sesquiterpenoids, diterpenoids, quassinoids, steroids, triterpenoids, simple and complex phenolics yielded by higher plants (Chitwood, 2002).

A notable nematicidal impact of the biotest solutions used in this study was that the exposure time played no role in the efficacy of *C. myriocarpus* biotest solutions. In another study, 50% of *C. elegans* were dead 2 hours after exposure (Sparg *et al.*, 2001). Extracts of *C. myriocarpus* are known to be highly toxic and are believed to be the bitterest of all known biochemical compounds (Jeffery, 1987; Rimington, 1998). Subcutaneous injection of cattle in an Australian study, resulted in the death of all treated cattle within 24 hours (McKenzie, Newman, Rayner and Dunster, 1988).

The most common extractible biochemical compounds by solvents used in this study are flavonoids, terpenoids, tannins, alkaloids, saponins and lutiolin (Appendix 1). Thus, the search for the nematicidal compound in *C. myriocarpus* biotest solutions should focus on these six compounds. In this study, P_1 (total ethanolic extract) and P_2 (100% hexane fraction) showed consistent high nematicidal effects on both *T. semipenetrans* and *M. incognita* studies, suggesting that they contain the potent chemical compounds which are being sought for in the VLIR Nematology Laboratory.

Cucumis myriocarpus fruits contain large quantities of highly toxic cucumis ($C_{27}H_{40}O_9$) and leptoderms ($C_{27}H_{38}O_8$), which are collectively called cucurbitacins (Van Wyk *et al.*, 1997). However, the specific component of *C. myriocarpus* fruits responsible for nematicidal impact is not yet known. The six compounds that are common in P_1 and P_2 biotest solutions (flavonoids, terpenoids, tannins, alkaloids, saponins and lutiolin), should provide some light on whether they contain cucurbitacins or not.

Bionematicidal impact on *M. incognita* and *T. semipenetrans* in this study confirm various studies that demonstrated consistent suppression of *M. incognita* by *C. myriocarpus* fruit amended soil under various conditions (Mabitsela, 2005; Mashela, 2002; Mphosi, 2004). *Cucumis myriocarpus* amended soil also increased the productivity of tomato.

In conclusion, the biotest solutions of *C. myriocarpus* resulted in higher nematode mortality than the non-biotest solutions. Because the chemical solvents were evaporated, it may be concluded that the high mortality in *M. incognita* and *T.*

semipenetrans were due to the chemical compounds from *C. myriocarpus* fruits. Regardless of the solvent used, antihelmintic properties were observed. However, total ethanolic extract and 100% hexane fraction were the best solvents for use in assessing antihelmintic properties of *C. myriocarpus* fruits.

CHAPTER 4

ANTIBACTERIAL EFFECTS OF *CUCUMIS MYRIOCARPUS*

4.1 Introduction

Bacillus species serve as effective microbes (EM) for organic decomposition (Todar, 2005). Commercially available *Bacillus* species comprise *B. litcheniformis*, *B. chitinosporus* and *B. laterosporus* (Mashela and Nthangeni, 2002). In various organic amendment studies, *Bacillus* species did not interact with the materials used (Mabitsela, 2005; Mphosi, 2004; Ngobeni, 2003).

Fruits of wild cucumber (*Cucumis myriocarpus*) contain cucurbitacins, which are highly toxic chemical compounds (Van Wyk *et al.*, 1997). Using *C. myriocarpus* as a bionematicide consistently reduced numbers of *Meloidogyne incognita* in tomato (Mabitsela, 2005; Mashela, 2002; Mphosi, 2004) and cowpea (Shakwane, 2005) production. *Bacillus* species did not interact with *C. myriocarpus* in nematode suppression, suggesting that *C. myriocarpus* extracts were toxic to *Bacillus* species (Mabitsela, 2005; Mphosi, 2004). The objective of this study was to determine the impact of *C. myriocarpus* biotest solutions on *Bacillus* species in bioactivity tests under laboratory conditions.

4.2 Materials and Methods

The experiment was initiated on 2 August 2004 in the Botany Microorganisms Laboratory, University of Limpopo (UL) and the Department of Botany, University of Pretoria (UP). Fruits of *C. myriocarpus* were locally collected, dried for 5 days in air-forced oven at 52^{o}C to minimize the loss of volatile phytochemicals and ground in a Wiley mill to pass through a 1-mm sieve.

Powdered fruit material (70.1 g) was extracted in 80% hexane: 20% dichloromethane, 80% hexane: 20% methanol, 20% hexane: 80% methanol, 100% dichloromethane, 80% hexane: 20% ethanol, 100% methanol, 100% acetone, 100% toluene, 100% water, 100% ethyl acetate, 100% petroleum ether and 100% hexane. Biotest extract of each solvent was filtered using Whatmann filter paper no. 1 and evaporated using a Rotavapor at 50°C. Prior to the bioassay, each extract was resuspended in DMSO, concentrated at 100 mg/ml and refrigerated at 4°C (Lall and Meyer, 2000; Rojas *et al.*, 2003; Sokmen *et al.*, 1998). The tested materials were (Dimethylsulphode) DMSO (B_2), used as the untreated control, 80% hexane: 20% dichloromethane (B_3), 80% hexane: 20% methanol (B_4), 20% hexane: 80% methanol (B_5), 100% dichloromethane (B_6), 80% hexane: 20% ethanol (B_7), 100% methanol (B_8), 100% acetone (B_9), 100% toluene (B_{10}), 100% water (B_{11}), 100% ethyl acetate (B_{12}), 100% petroleum ether (B_{13}), 100% hexane (B_{14}). Other materials and methods were as described for the University of Pretoria study (Chapter 3) and the biotest solutions were total ethanolic extract (P_1), 100% hexane fraction (P_2), hexane-ethyl acetate (1:1, v/v) fraction (P_3), 100% ethyl acetate fraction (P_4) and 100% methanol fraction (P_5).

The agar-well diffusion method was used to determine the growth-inhibition of *Bacillus* species by *C. myriocarpus* extracts (Perez, Paul and Bazerque, 1990). Two litter Mueller-Hinton nutrient agar was autoclaved at 121°C for 30 minutes and then poured into 100-ml x 15-ml sterile Petri dishes in the Lamina flow system, and allowed to set. After setting, Petri dishes were closed and placed upside-down at 4°C in the refrigerator to ensure that moisture did not contaminate the agar. Biostart® (Microbial Solutions LTD, Strubens Valley, RSA), comprising a mixture of *B. chitinosporus*, *B.*

laterosporus and *B. litcheniformis*, was used at the strength of 10^9 CFU/ml. *Bacillus* species were cultured on nutrient agar and incubated at 37°C for 24 hours. The multiplied bacteria were stored at 4°C prior to use.

Working on the Lamina flow bench, *Bacillus* species were suspended in apprimately 200 ml of saline solution in the glass flask, standardized to McFarland 1 solution and uniformly spread on the surface of the agar using swab sticks. A 5-mm diameter sterile cork-borer was used to bore 4 wells in the set agar per Petri dish. Ten microliters of each biotest solution was added into each well and allowed to diffuse for one and half hours. Each Petri dish was closed and sealed using parafilm. The treatments were arranged in a completely randomized design in the growing chamber at 37°C. Each treatment was replicated 5 times. The experiment was terminated after 24 hours.

The degree of the bioactivity of the biotest solution was indicated by the presence or absence of clear zone around the wells. A transparent ruler was placed at the centre of the well to record the diameter (mm) of the inhibition zone. An average of five replications was recorded. Data were analysed using analysis of variance (ANOVA) and when the treatment means were different ($P \leq 0.05$), mean separation was done using the Duncan multiple range test for University of Limpopo biotest solutions and using Least significant difference (LSD) for the University of Pretoria biotest solutions (Gomez and Gomez, 1984). Means were reported in tables and also using column charts.

4.3 Results

The bioassays observed suggested that irrespective of whether *C. myriocarpus* was in extract or fraction form, the material inhibited growth of EM. In Experiment 1 and Experiment 3, the observations in the inhibition of the growth of EM were clear. However, in Experiment 2, the observations were not as clear as in Experiment 1 and Experiment 3. Bacterial growth inhibition under the bioassayed materials differed from that of the control.

Presence of clear zones is an indication of inhibition of bacterial growth by the biotest solution, whereas absence is an indication of the inactivity of the tested solution (Figure 4.1).

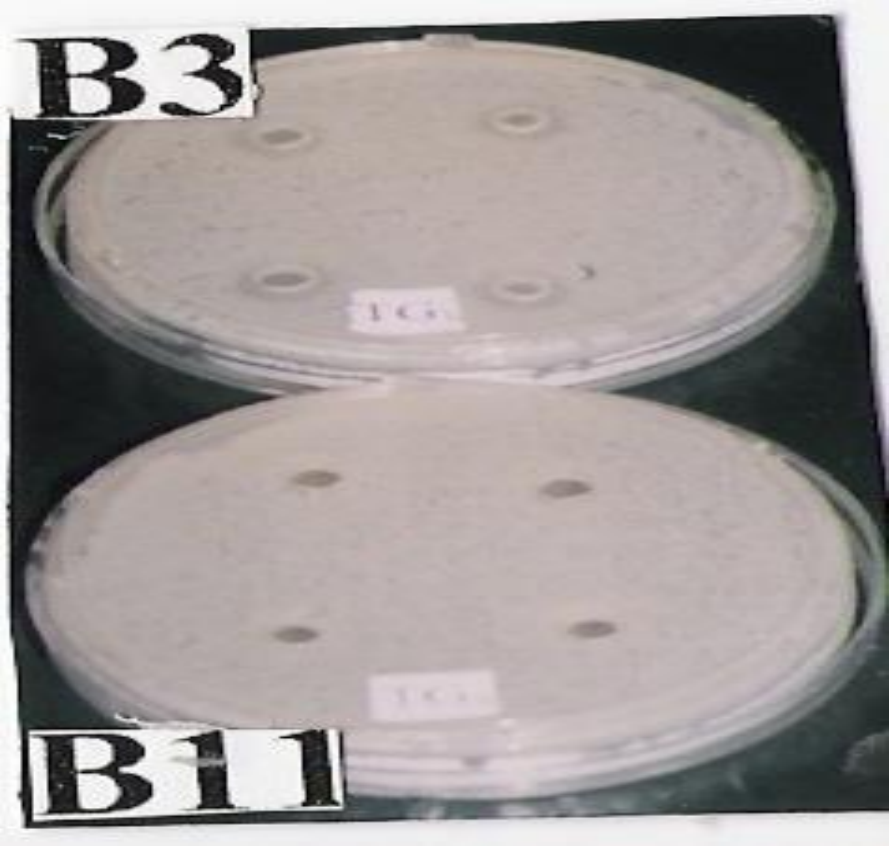


Figure 4.1 Illustration of inhibition zones in 80% hexane: 20% dichloromethane (B_3) and 100% water (B_{11}) biotest solutions

4.3.1 Biotest solutions from University of Limpopo

The B_{11} and B_2 biotest and non-biotest solutions of *C. myriocarpus*, respectively, did not inhibit growth of the EM. The B_3, B_4, B_5, B_6, B_7, B_8, B_9, B_{10}, B_{12}, B_{13} and B_{14} biotest solutions of *C. myriocarpus* inhibited growth of EM, however, the means of the inhibition zones varied among treatments.

Table 4.1 Bacterial growth inhibition zones of *Cucumis myriocarpus* in eleven solvents

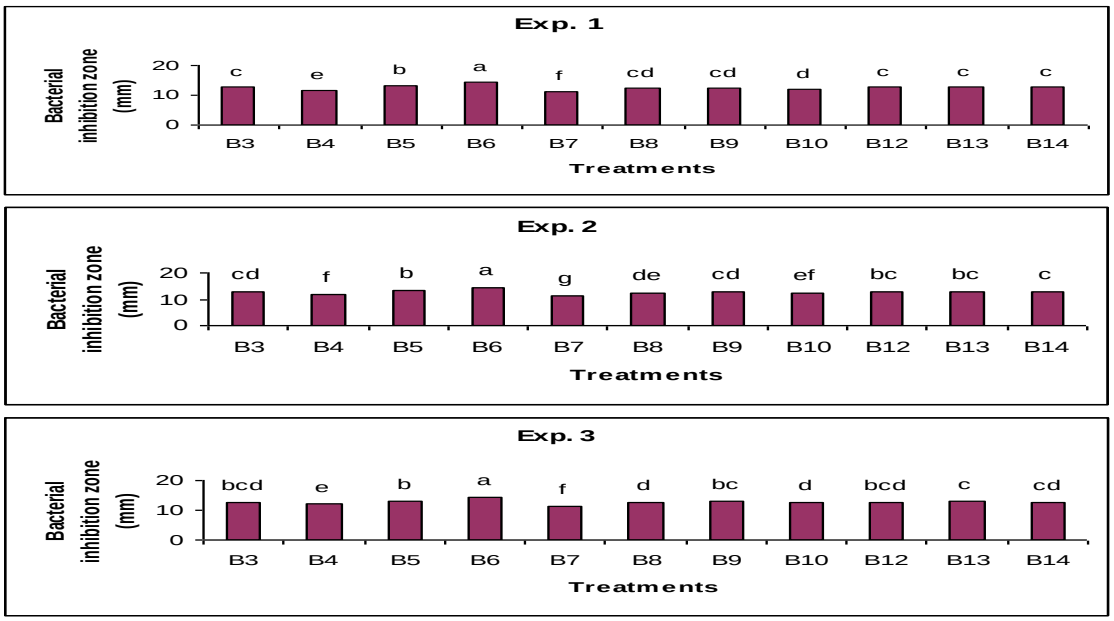
Treatment	N	Inhibition zone (mm)					
		Experiment 1		Experiment 2		Experiment 3	
B_3	20	12.65	c	12.70	cd	12.80	bcd
B_4	20	11.80	e	11.90	f	12.05	e
B_5	20	13.20	b	13.25	b	13.15	b
B_6	20	14.35	a	14.60	a	14.35	a
B_7	20	11.40	f	11.50	g	11.50	f
B_8	20	12.50	cd	12.35	de	12.50	d
B_9	20	12.50	cd	12.70	cd	13.10	bc
B_{10}	20	12.20	d	12.20	ef	12.55	d
B_{12}	20	12.65	c	12.90	bc	12.80	bcd
B_{13}	20	12.70	c	13.00	bc	13.00	c
B_{14}	20	12.80	c	12.85	c	12.70	cd

Column means with the same letter were not different ($P \leq 0.05$) according to Duncan's multiple range test.

B_3 = 80% hexane: 20% dichloromethane; B_4 = 80% hexane: 20% methanol; B_5 = 20% hexane: 80% methanol; B_6 = 100% dichloromethane; B_7 = 100% ethanol; B_8 = 100% methanol; B_9 = 100% acetone; B_{10} = 100% toluene; B_{12} = 100% ethyl acetate; B_{13} = 100% petroleum ether; B_{14} = 100% hexane.

In about 24 hours after initiating the treatments the bioactivity of the extract (B_6) on the growth of EM was consistent and differed from others in all three experiments (Figure 4.1). The effect of the extract (B_5) on EM growth inhibition was consistent in all experiments, however, it differed from all other extracts in Experiment 1 and did not differ with those of the extracts (B_{12} and B_{13}) in Experiment 2 and with those of extracts (B_3, B_9, B_{12} and B_{13}) in Experiment 3.

The bioactivity of the extract (B_3) on EM did not differ with those of the extracts (B_8, B_9, B_{12}, B_{13} and B_{14}) in Experiment 1 and Experiment 3 but differed with that of the extract (B_8) in Experiment 2. Although the bioactivity of the extract (B_{10}) against EM was inconsistent, it did not differ with those of the extract (B_8) in Experiment 1 and Experiment 3, and that of the extract (B_9) in Experiment 1 and that of the extract (B_3, B_{12} and B_{14}) in Experiment 3. The effect of the extract (B_4) differed from those of all other extracts, however, it was not different from that of the extract (B_{10}) in Experiment 2. Although not different from the extract (B_{10}) in Experiment 2, the bioactivity of the extract (B_7) on EM differed from all other extracts. The effects of the DMSO (B_2) and the extract (B_{11}) did not differ in all the experiments.



B_3 = 80% hexane: 20% dichloromethane; B_4 = 80% hexane: 20% methanol; B_5 = 20% hexane: 80% methanol; B_6 = 100% dichloromethane; B_7 = 100% ethanol; B_8 = 100% methanol; B_9 = 100% acetone; B_{10} = 100% toluene; B_{12} = 100% ethyl acetate; B_{13} = 100% petroleum ether; B_{14} = 100% hexane.

Figure 4.2 Effects of biotest solutions of *Cucumis myriocarpus* fruits on effective microbes over 24 hours

4.3.2 Biotest solutions from University of Pretoria

The P_1, P_2, P_3, P_4 and P_5 biotest solutions of *C. myriocarpus* were tested for the antibacterial activity. The growth of the EM under study was inhibited by all biotest

solutions, however, the reported means of the inhibition zones differed from one treatment to another.

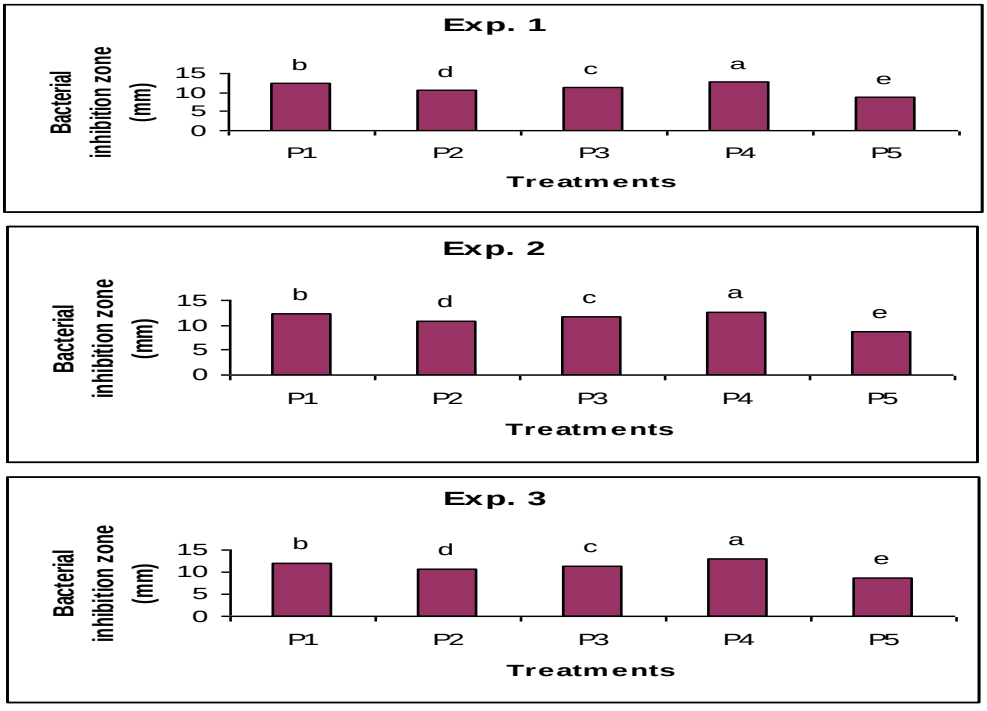
Table 4.2 Bacterial growth inhibition zones of *Cucumis myriocarpus* in five solvents

Treatment	N	Inhibition zone (mm)		
		Experiment 1	Experiment 2	Experiment 3
P_1	5	12.30b	12.35b	12.10b
P_2	5	10.55d	10.75d	10.60d
P_3	5	11.40c	11.70c	11.45c
P_4	5	12.80a	12.75a	12.85a
P_5	5	8.80e	8.70e	8.65e

Column means with the same letter were not different ($P \leq 0.05$) according to Duncan's multiple range test.

P_1 = total ethanolic extract; P_2 = 100% hexane fraction; P_3 = hexane-ethylacetate (1:1, v/v) fraction; P_4 = 100% ethylacetate fraction and P_5 = 100% methanol fraction.

In about 24 hours after initiating treatments, the bioactivity of the biotest solutions on the inhibition of the growth of EM differed from one another in all experiments (Figure 4.2).



P_1 = total ethanolic extract; P_2 = 100% hexane fraction; P_3 = hexane-ethylacetate (1:1, v/v) fraction; P_4 = 100% ethylacetate fraction and P_5 = 100% methanol fraction.

Figure 4.3 Effects of biotest solutions of *Cucumis myriocarpus* fruits on effective microbes over 24 hours.

4.4 DISCUSSION

Biotest solutions from *C. myriocarpus* extracts and fractions demonstrated that *C. myriocarpus* has antibacterial properties. Although the various solvents used in this study have the ability to extract chemical compounds from plant tissues, the extractible chemical compounds differ with respect to the solvent used (Appendix 1). Some common extractable chemical compounds exist among solvents used in this study. The activity of *C. myriocarpus* extracts and fractions on inhibition of *Bacillus* species confirm various studies where significant interactions between *Bacillus* species and *C. myriocarpus* could not be demonstrated (Mabitsela, 2005; Mphosi, 2004).

Chemical compounds from various plants, extracted by the solvents used in this study, have demonstrated the ability to inhibit growth of various bacteria, which most of them were disease causal agent to human beings. In this study, extracts from 100% dichloromethane and 20% hexane: 80% methanol were the most potent. A strong demonstration was displayed by the methanol extract from *Kielmeyera varebilis*, *Helenium donianun*, *Toona ciliate*, *Amoora rohituka* and *Vernonia cinerea* (Chowdhury, Hasan and Rashid, 2002; Feresin, Tapia and Bustos, 1999; Gupta, Mazumder, Manikandan, Haldar, Buttacharya and Kandar, 2002; Pinheiro *et al.*, 2003). Dichloromethane and petroleum ether biotest solutions from *Nepeta cataria* flowers, *T. ciliate*, *C. facultus*, *C. suaveolens and C. mackenii* also showed strong activity against *B. subtilis* (Chowdhury *et al.*, 2002; Elgorashi and Van Staden, 2003; Sparg *et al.*, 2001). This is the first antibacterial study on extracts from fruits of *C. myriocarpus*.

The biotest solution from *Helichrysum italicum*, *T. ciliate*, *A. rohituka* and *Mapia foetida* extracted with petroleum ether showed strong activity against *B. subtilis*, *B.*

cereus and *B. megaterium* (Chowdhury *et al.*, 2002; Hossain, Paul, Sorab, Rahman and Rashid, 2001; Nostro *et al.*, 2000). The ethanol biotest from *Salvia officinalis*, *Anthocleista djalonensis*, *Nauclea latifolia*, *Uvaria afzalii*, *S. natalensis*, *L. ovatifolia* and *Vitex trifolia* were found to be active against *B. subtilis*, *B. cereus* and *B. megaterium* (Hossain *et al.*, 2001; Okoli and Iroegbu, 2004; Sparg *et al.*, 2001; Velickovic *et al.*, 2003). The biotest solution from the roots of *A. bracteata*, roots of *Euclea natalensis*, *Azadirachta indica* and *Ruta graveolens* extracted with acetone inhibited growth of *B. cereus*, *B. pumilus* and *B. subtilis* (Alzoreky and Nakahara, 2002; Lall and Meyer, 2000; Negi *et al.*, 2003).

Bacillus subtilis growth was inhibited by the biotest solutions from *P. reglallii*, roots of *A. bracteata*, *C. nutans*, *Terminalia arjuna* and *Proteus vulgaris* extracted using ethyl acetate (Negi *et al.*, 2003; Pessini *et al.*, 2003; Samy, Ignacinuthu and Sen, 1998; Truiti *et al.*, 2003). *Bacillus subtilis* was also inhibited by the toluene biotest solution from the leaves of *Phyllanthus emblica* (Summanen, 1999). Inhibition of *B. subtilis* was also shown by biotest solutions from *P. regnellii*, *K. variabilis*, *Lippia graveolans*, *M. parviflora* and *C. nutans* using hexane (Elvin-Lewis, 2001; Fugh-Berman, 2000; Hernandez *et al.*, 2003; Pessini *et al.*, 2003; Pinheiro *et al.*, 2003; Truiti *et al.*, 2003).

Synergism of two or more plant chemical compounds is key to the observed bioactivities. However, sufficient quantities of the chemical should be extracted for the activity to manifest (Alzoreky and Nakahara, 2002; Fugh-Berman, 2000). Generally, when insufficient quantities of plant chemical compounds are extracted and when synergism criterion is not met between two or more compounds, bioactivity may not manifest. Good examples were those of methanol biotest solutions of *C. facultus*,

C. suaveolens and *C. mackenii* and n-hexane biotest solution of *Scilla natalensis* and *Ledebouria ovatifolia* where bioactivities were not observed, and the water biotest solution of *Piper regenii* that showed poor activity against *B. subtilis* (Elgorashi and Van Staden, 2003; Pessini *et al.*, 2003; Sparg *et al.*, 2001). The cited bioactivity tests were conducted on *Bacillus* species, which are all gram positive. In some of the studies where gram negative and positive bacteria were included, the plant materials tested had variable effects on the test organisms, with gram negative bacteria showing some resistance (Kelmanson, Jager and Van Staden, 2000). The presence of resistance on gram negative bacteria was due to the cell wall that is surrounded by an extra layer of polysaccharides, proteins and phospholipids (Porter, 1998).

In conclusion, the biotest solutions of *C. myriocarpus* fruits resulted in the inhibition of *Bacillus* species. Because of evaporation of the biotest solutions, it can be concluded that bacterial inhibition was due to the chemical compounds from *C. myriocarpus* per se. In all tests, 100% dichloromethane (B_6) and 20% hexane: 80% methanol (B_5) had the highest growth inhibition of *Bacillus* species.

CHAPTER 5

MINIMUM INHIBITORY CONCENTRATION OF *CUCUMIS MYRIOCARPUS* ON *BACILLUS* SPECIES

5.1 Introduction

The minimum inhibitory concentration (MIC) is the lowest concentration of an antibiotic required to inhibit the growth of an organism *in vitro* (Alzoreky and Nakahara, 2003; Jacobs, DeMott, Finley, Horvak, Kasten and Tilzer, 1994). Every chemical designed to kill an organism has MIC at which it kills the organism. Different extracts from plants may vary in the MIC required to kill a given organism (Kianbakht and Jahaniani, 2003). Previously, biotest solutions of wild cucumber fruits (*Cucumis myriocarpus*) demonstrated antibacterial effects on *Bacillus* species (Chapter 4). The MIC of *C. myriocarpus* on *Bacillus* species has not been documented. The objective of this study was to determine the MICs of various *C. myriocarpus* biotest solutions.

5.2 Materials and Methods

The MIC study was conducted on 18 August 2004 at the Department of Botany Microorganisms Laboratory, University of Limpopo (UL). Fruits of *C. myriocarpus* were locally collected, dried for 5 days in air-forced oven at 52 ^{o}C to minimize the loss of volatile phytochemicals and ground in a Wiley mill to pass through a 1-mm sieve.

Powdered fruit material (70.1 g) was extracted with 12 different solvents, namely, 80% hexane: 20% dichloromethane, 80% hexane: 20% methanol, 20% hexane: 80% methanol, 100% dichloromethane, 80% hexane: 20% ethanol, 100% methanol, 100% acetone, 100% toluene, 100% water, 100% ethyl acetate, 100% petroleum ether and

100% hexane. Biotest extracts of each solvent were filtered using Whatmann filter paper no. 1 and the filtrate evaporated using a Rotavapor at 50°C. Each extract was then resuspended in (Dimethylsulphoxide) DMSO, concentrated to 100 mg/ml and refrigerated at 4°C (Lall and Meyer, 2000; Rojas *et al.*, 2003; Sokmen *et al.*, 1998). The UL biotest solutions included the untreated control, DMSO (B_2), 80% hexane: 20% dichloromethane (B_3), 80% hexane: 20% methanol (B_4), 20% hexane: 80% methanol (B_5), 100% dichloromethane (B_6), 80% hexane: 20% ethanol (B_7), 100% methanol (B_8), 100% acetone (B_9), 100% toluene (B_{10}), 100% ethyl acetate (B_{12}), 100% petroleum ether (B_{13}), 100% hexane (B_{14}). In another study extracts prepared at the University of Pretoria (UP) were used (Chapter 4). The biotest solutions included total ethanolic extract (P_1), 100% hexane fraction (P_2), hexane-ethyl acetate (1:1, v/v) fraction (P_3), 100% ethyl acetate fraction (P_4) and 100% methanol fraction (P_5). The biotest solutions were prepared using 500 g ground *C. myriocarpus* fruit.

The microtiter plates were placed on the Laminaflow bench, and 100 µl nutrient broth pipetted into the wells. Biotest solutions were serially pipetted at 100 µl into microplates (Eloff, 1998; Reiner, 1982; Rhajaoui *et al.*, 2001). Each 100 µl contained 100 mg/ml of plant extract, and adequate mixing with ensured by pulling-and-releasing the pipetted solution five times. The concentration of each biotest solution was decreased by half from one well to the next, with final concentrations per solution being 0.01, 0.02, 0.05, 0.10, 0.20, 0.39, 0.78, 1.56, 3.13, 6.25, 12.5 and 25 mg *C. myriocarpus* extract. Biostart® (Microbial Solutions LTD, Strubens Valley, RSA), comprising a mixture of *B. chitinosporus*, *B. laterosporus* and *B. litcheniformis* was used at the strength of 10^9 CFU/ml, mixed with 100-ml saline solution in a glass flask, and standardised to McFarland 1 solution (Border and Fireham mer, 1980). The aliquot

was added into the microplates at 100 µl, with microplates lids being tightly sealed with parafilm to eliminate contamination. Each treatment was replicated four times and microplates were incubated for 24 hours at 37 °C.

The p-iodonitrotetrazolium violet (INT) solution at 0.2 mg/ml was added into each well at 50 µl and re-incubated at 37 °C for 30 minutes (Reiner, 1982). Because *Bacillus* species are gram-positive (Dib, Dib, Korkmaz, Mobarakai and Glaser, 2003), the development of purple colour in the wells indicated effective bacterial growth, whereas absence of colour was indicative of inhibition of bacterial growth. The concentration of the biotest solution which precedes the one with purple colour is the MIC for that solution (Bylka, Szaufer-Hajdrych, Matlawska and Goslinska, 2004). The MICs for various biotest solutions were recorded.

5.3 Results

The bioactivity of the biotest solutions B_{11} and B_{12} inhibited bacterial growth at MIC values of 3.13 mg/ml, whereas B_3, B_5, B_6, B_8, B_9, B_{10} and B_{14} inhibited *Bacillus* growth at 6.25 mg/ml (Table 5.1). The biotest solutions B_4 and B_7 showed bioactivity at 12.5 mg/ml. Bacterial growth occurred in all wells of B_2, which was an untreated control.

The P_3 and P_4 biotest solutions inhibited bacterial growth at 0.78 mg/ml, whereas P_1 and P_2 at 1.56 mg/ml (Table 5.2). Although *C. myriocarpus* used in this study (UP) was quite higher than that at UL, 100% methanol in both studies (B_8 and P_5) had the same MIC values of 6.25 mg/ml, whereas for other biotest solutions the values differed.

Table 5.1 Minimum inhibitory concentrations (MIC) of *Cucumis myriocarpus* biotest solutions as depicted by inhibition of bacterial growth (UL)

Solvent	Biotest solution	Biotest solution with bacterial growth (mg/ml)	Biotest solution without bacterial growth (mg/ml)
Dimethylsulphoxide	B_2	25.00	-
80% hexane: 20% dichloromethane	B_3	3.13	6.25
80% hexane: 20% methanol	B_4	6.25	12.50
20% hexane: 80% methanol	B_5	3.13	6.25
100% dichloromethane	B_6	3.13	6.25
100% ethanol	B_7	6.25	12.50
100% methanol	B_8	3.13	6.25
100% acetone	B_9	3.13	6.25
100% toluene	B_{10}	3.13	6.25
100% ethyl acetate	B_{12}	1.57	3.13
100% petroleum ether	B_{13}	1.57	3.13
100% hexane	B_{14}	3.13	6.25

Table 5.2 Minimum inhibitory concentrations (MIC) of *Cucumis myriocarpus* biotest solutions as depicted by inhibition of bacterial growth (UP)

Solvent	Biotest solution	Biotest with bacterial growth (mg/ml)	Biotest without bacterial growth (mg/ml)
Dimethylsulphoxide	B_2	25.00	-
total ethanolic extract	P_1	0.78	1.56
100% hexane	P_2	0.78	1.56
hexane-ethyl acetate (1:1 v/v)	P_3	0.39	0.78
100% ethyl acetate	P_4	0.39	0.78
100% methanol	P_5	3.13	6.25

5.4 DISCUSSION

Biotest solutions of *C. myriocarpus* fruit inhibited growth of *Bacillus* species at low MIC value. Results of this study confirm the antibacterial activities of *C. myriocarpus* that were observed previously (Chapter 4).

Various studies demonstrated that *Bacillus* species were not essential for the efficacy of ground *C. myriocarpus* to suppress *Meloidogyne incognita* under both greenhouse and field studies (Mabitsela, 2005; Mphosi, 2004). Antibacterial activities in this study provide a clue as to why there were no *Cucumis* x *Bacillus* interactions in the cited studies. Also, when using castor bean (*Ricinus communis*) fruits, *Ricinus* x *Bacillus* interactions were not significant ($P \leq 0.05$) in suppression of *M. incognita* numbers (Mabitsela, 2005; Mashela and Nthangeni, 2002). However, not all materials used showed this trend. Mangena (2005) demonstrated that *Brassica* x *Bacillus* interactions were significant ($P \leq 0.05$) in suppression of *M. incognita* numbers.

Results of this study confirm the ground-leaching technology (Mashela, 2002) which was developed in an attempt to ameliorate the disadvantages of conventional application of organic matter (Stirling, 1991). Briefly, the technology involves using small quantities of toxic organs in powdered form to suppress plant-parasitic nematodes. Mashela (2002) suggested that microbial decomposition was not essential for the release of nematicidal compounds in this technology, and that the compounds were leached out of organic matter through irrigation water.

The impact of *C. myriocarpus* on *Bacillus* species would probably not negate the chances of its future development into a commercial product. Generally, *Bacillus* species are gram-positive, whereas most gram-negative bacteria that occur in the soil have better resistance against chemicals. For instance, the use of *C. myriocarpus* in cowpea production improved nodulation (Shakwane, 2005). Thus, it appears that not all bacteria are negatively impacted by this material. Also, it is common knowledge that bacteria constitute a small fraction of microbial decomposing organisms (Cottrell

and Kirchman, 2000). Effective microbes consist of photosynthetic bacteria, lactic acid, yeast, fungi and *actinomycetes*.

CHAPTER 6

SUMMARY AND CONCLUSION

Ethanolic extract and 100% hexane fraction were the best solvents for extracting antihelmintic chemical compounds from wild cucumber (*Cucumis myriocarpus*) fruits, whereas 100% dichloromethane and 20% hexane: 80% methanol extracts were the best for extracting antibacterial chemical compounds. Other used chemical solvents also extracted both antihelmintic and antibacterial chemical solvents from fruits of *C. myriocarpus*.

Commonly extractible chemical compounds in ethanol, hexane and dichloromethane are flavonoids, terpenoids, phenols, tannins and alkaloids (Appendix 1). Two cucurbitacins, cucumins ($C_{27}H_{40}O_9$) and leptodermins ($C_{27}H_{38}O_8$) are known to be the toxic components of *C. myriocarpus* fruits (Van Wyk *et al.*, 1997). The chemical compound, cucumis, is a flavonoid (Krauze-Baranowska and Cisowski, 2001).

Results of this study also confirmed the antihelmintic properties of *C. myriocarpus* fruits reported under greenhouse, microplot and field studies (Mabitsela, 2005; Mashela, 2002; Mphosi, 2004). Plants of *C. myriocarpus* are non-host to *Meloidogyne incognita* (Mofokeng, 2005), which confirm reports that indicate that cucurbitacins accumulate in both fruits and roots (Van Wyk *et al.*, 1997).

The antibacterial properties observed in this study may confirm the absence of interactions between *Bacillus* species and ground *C. myriocarpus* fruits under microplot and field studies (Mabitsela, 2005; Mphosi, 2004). Also, the minimum inhibitory concentration of *C. myriocarpus* fruit extracts is quite low, confirming

reports which categorise extracts from this fruit as being highly toxic (Van Wyk *et al.*, 1997).

In ground form, *C. myriocarpus* fruits are used in small quantities, for instance 0.71 tons/ha. When used in these small quantities, *C. myriocarpus* fruits improved the efficacy of *Rhizobium* species in cowpea (Shakwane, 2005). Thus, the impact of *C. myriocarpus* fruit on non-target organisms such as *Bacillus* species which are responsible for decomposition require additional studies under various soil conditions.

In conclusion, the solvents which should be used for characterising nematicidal compounds in *C. myriocarpus* fruits are ethanol and 100% hexane. However, water is also capable of extracting chemical compounds that are extracted by ethanol and 100% hexane from plant materials.

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APPENDICES

Appendices 1: Extractible plant compounds by solvents used in this study

Solvent	Extractible plant compounds	Commonly extractible compounds
Water	Phenolics, flavonoids, tannins, terpenoids, saponins.	Flavonoids, terpenoids, tannins, phenolics
Ethanol	Terpenoids (diterpenoids, monoterpenoids), heleanolides, guaianolides, pseudogluaianolides, flavonoids, coumarines, sesquiterpenoids, tannins, saponins, steroids.	Flavonoids, terpenoids
Hexane	Phenolics, flavonoids, kaenpferol, lutiolin, palargonidin, oleic acid, trans-asarone, trimethoxybenzaldehyde, geraniol, Alkaloids, saponins, glycosides.	Flavonoids, Phenolics, Alkaloids, saponins
Ethyl acetate	Alkaloids, flavonoids, tannins, terpenoids, saponins, sterols, triterpenoids, lutiolin, polyphenolics, arjunolic acid.	Flavonoids, terpenoids, tannins, alkaloids
Methanol	Cardenolides, flavonoids (flavones), phenols, alkaloids, tannins, terpenoids (triterpenoids), saponins.	Flavonoids, terpenoids, tannins, saponins
Petroleum ether	Steroids, terpenoids, flavonoids, tannins, naphthoquinones, triterpenoids, saponin, glycoside, sterols.	Flavonoids, terpenoids, tannins, saponins
Toluene	Alkaloids, benzonoid, furanolactone, carbohydrate, diterpene, triterpenes, flavonoids, sterols.	Flavonoids, alkaloids
Acetone	Naphthoquinones, terpenoids (triterpenoids), tannins.	Terpenoids, tannins
Dichloromethane	Limonoids, chrome, flavonoids, coumarins, alkaloids, terpenoids, tritepenes, diterpene.	Flavonoids, terpenoids, alkaloids

Source: (Ankli, Heimann, Heinrich and Sticher, 2000; Chatterjee, Kundu, Chakrabortty and Chandrasekharan, 1970; Chowdhury, Hasan and Rashid, 2002; DaSilva, Agostinho, Paula, Neto, Gamboa and Filho, 1999; Feresin, Tapia and Bustos, 1999; Gupta, Mazumder, Manikandan, Haldar, Buttacharya and Kandar, 2002; Hossain, Paul, Sorab, Rahman and Rashid, 2001; Kumar, Vishwanathan, Suresh and Mohan, 2002; Lall and Meyer, 2000; Neto, Owens, Langfield, Comeau, Onge, Vaisberg and Hammond, 2002; Okoli and Iroegbu, 2003; Prashanth, Asha and Amit, 2001; Sparg, Van Staden and Jager, 2001;).

Appendix 3.1 Number of dead *Meloidogyne incognita* in every second day after treatment initiation for 8 days

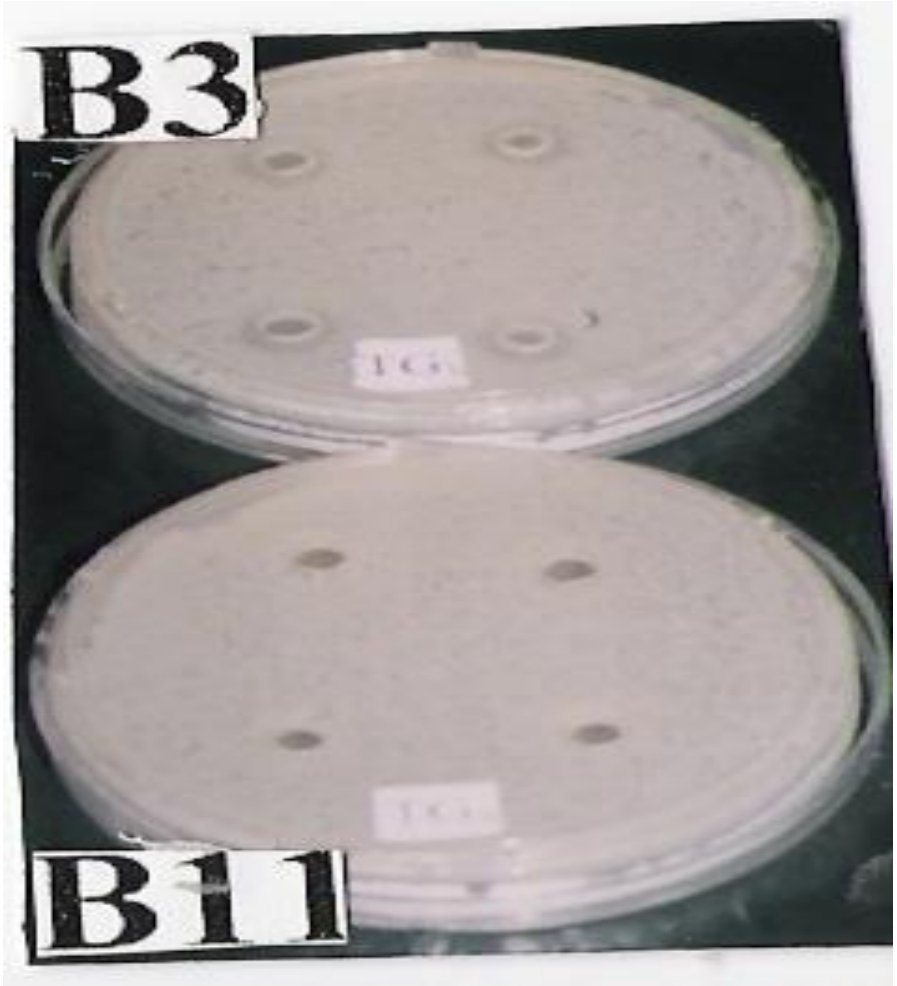
Day	Fraction1		Fraction2	Fraction3	Fraction4	Fraction5	Water	DMSO	
2	100.50	ab	131.75a	122.75a	134.00a	119.25a	48.25b	34.00	ab
4	138.00	a	133.25a	142.50a	147.75a	126.25a	33.75b	32.00	b
6	131.75	a	152.25a	151.50a	150.25a	127.75a	28.00b	33.50	b
8	146.50	a	173.25a	175.50a	175.25a	129.75a	36.00b	27.50	b

Appendix 3.2 Mortality of *Tylenchulus semipenetrans* eight days after exposure to biotest solutions

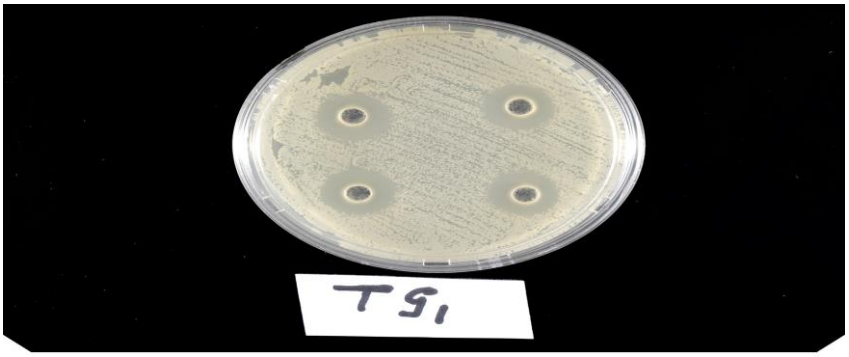
Experi	Treatment						
ment	P_1	P_2	P_3	P_4	P_5	B_1	B_2
1	1466.70a	1600.50a	1815.00a	1747.20a	738.83b	251.17c	273.17c
2	40.25abc	89.75a	83.75a	62.75abc	72.25ab	11.25c	20.50bc

B_1 = water; B_2 = DMSO; P_1 = total ethanolic extract; P_2 = 100% hexane fraction; P_3 = hexane-ethyl acetate (1:1, v/v) fraction; P_4 = 100% ethyl acetate fraction; P_5 = 100% methanol fraction.

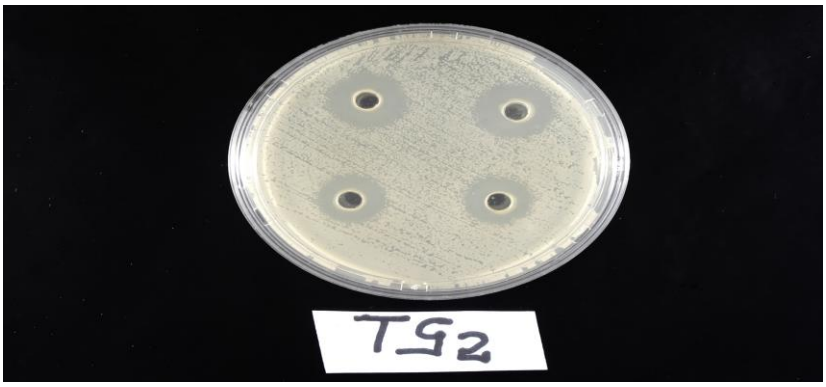
Appendix 4.1 Bioactivity of B_3, and inactivity of B_{11} against effective microbes



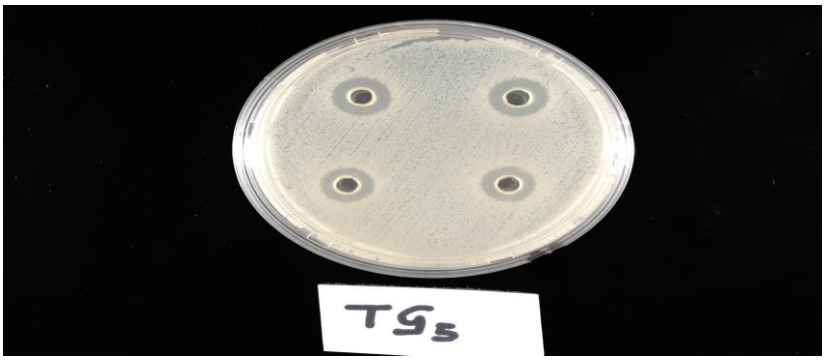
Appendix 4.2 Bioactivity of B_3 against effective microbes



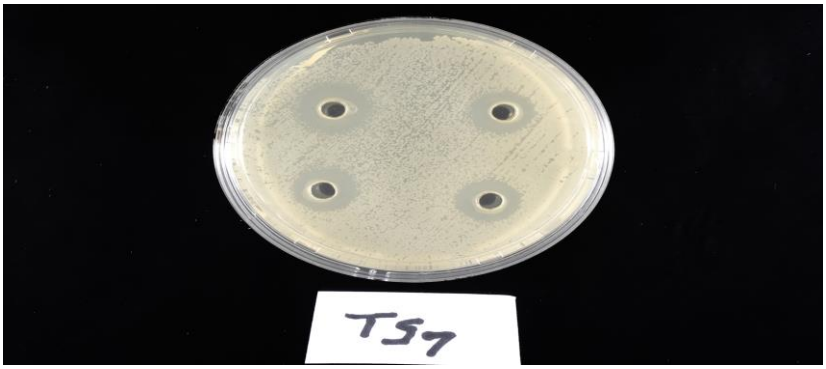
Appendix 4.3 Bioactivity of B_4 against effective microbes



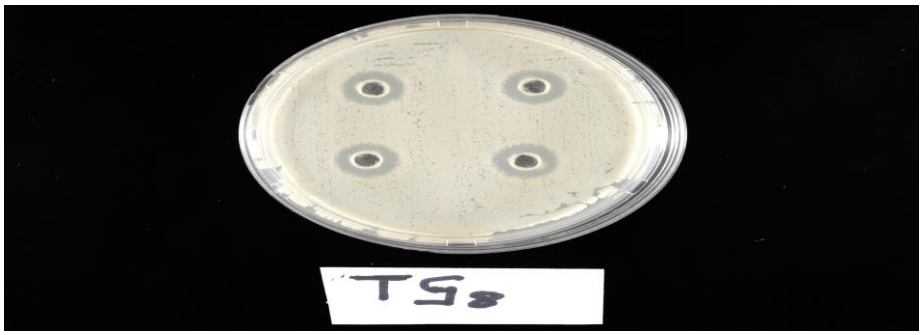
Appendix 4.4 Bioactivity of B_7 against effective microbes



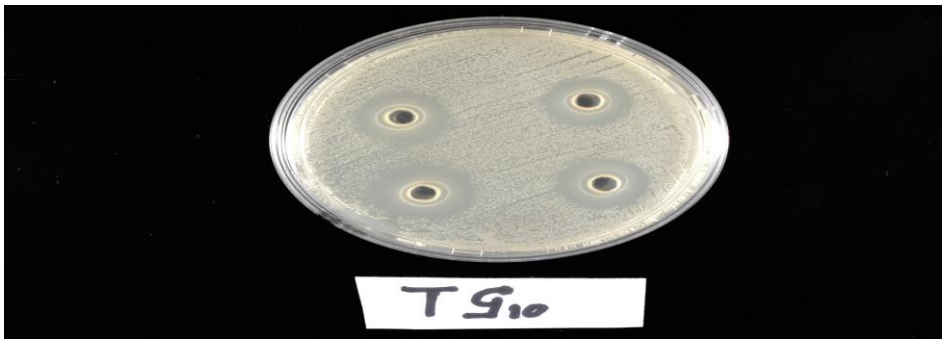
Appendix 4.5 Bioactivity of B_9 against effective microbes



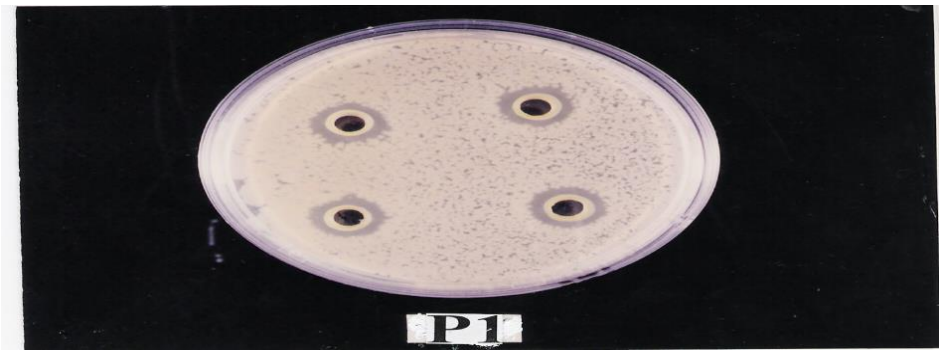
Appendix 4.6 Bioactivity of B_{10} against effective microbes



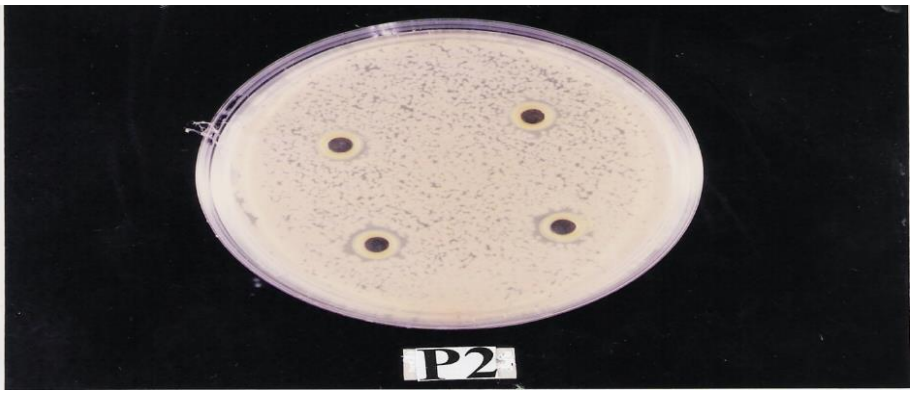
Appendix 4.7 Bioactivity of B_{12} against effective microbes



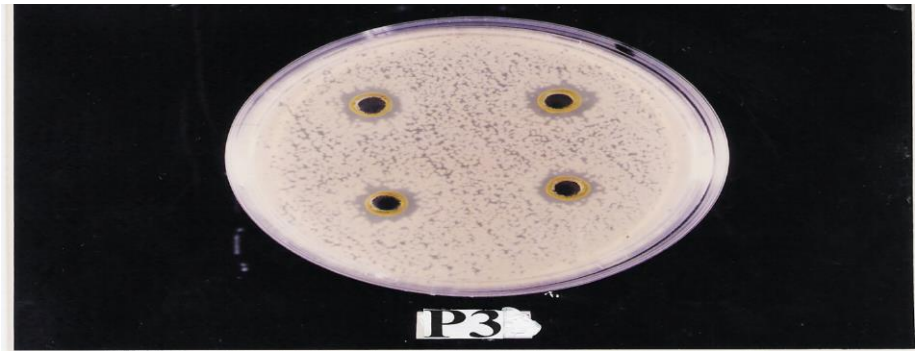
Appendix 4.8 Bioactivity of P_1 against effective microbes



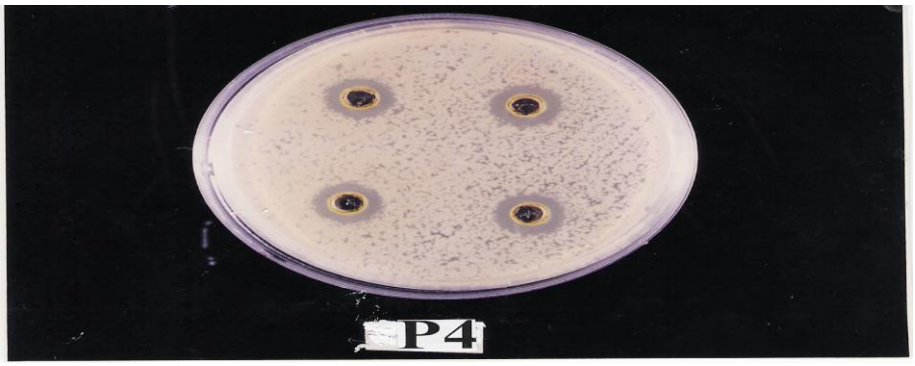
Appendix 4.9 Bioactivity of P_2 against effective microbes



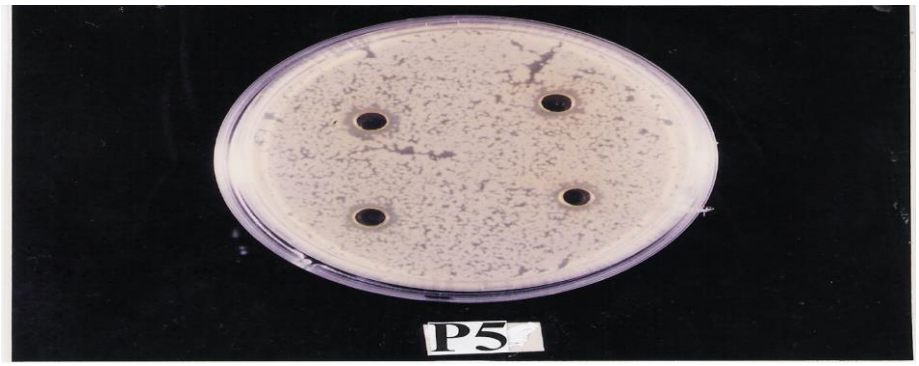
Appendix 4.10 Bioactivity of P_3 against effective microbes



Appendix 4.11 Bioactivity of P_4 against effective microbes

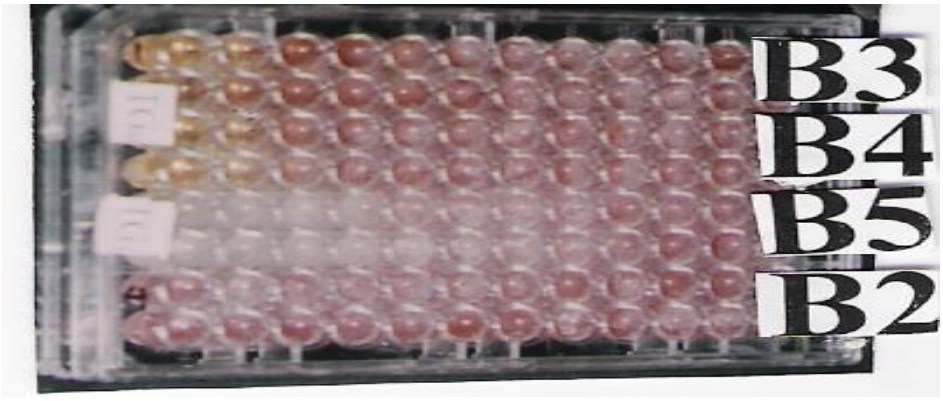


Appendix 4.12 Bioactivity of P_5 against effective microbes

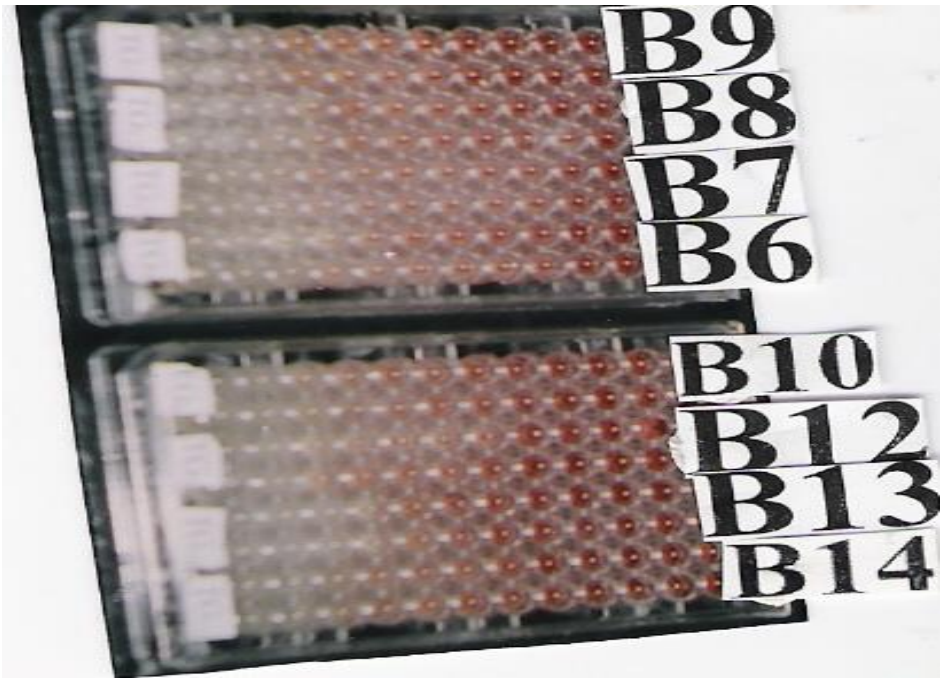


Appendix 4.13 Inactivity of B_2 against effective microbes

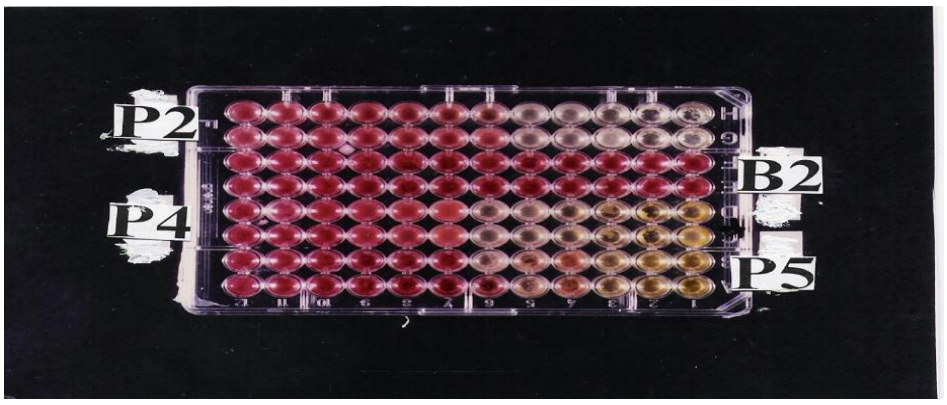
Appendix 4.14 Minimum inhibitory concentration of four biotest solutions (UL_1)



Appendix 4.15 Minimum inhibitory concentration of eight biotest solutions (UL_2)



Appendix 4.16 Minimum inhibitory concentration of four biotest solutions (UP_1)



Appendix 4.17 Minimum inhibitory concentration of four biotest solutions (UP_2)