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Abstract

Thirteen South African medicinal plants which are used traditionally to treat symptoms associated with *Listeria monocytogenes* infections, were screened for activity against the pathogen. Different plant parts were extracted separately with ethyl acetate or chloroform. All the extracts were first screened against the bacteria using the disc diffusion method. Zones of inhibition observed in the presence of the chloroform extracts of *Eucomis autumnalis*, ethyl acetate extracts of *Acacia karroo* and *Plectranthus ecklonii* (50 mg/ml) were 12 mm, 14 mm and 15 mm respectively. Active extracts were further tested against the bacteria for minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) using the microtitre dilution method. Ethyl acetate extracts of *A. karroo* exhibited MIC of 3.1 mg/ml and MBC of 6.25 mg/ml. Ethyl acetate extracts of *P. ecklonii* showed MIC of 0.5 mg/ml and MBC of 1.0 mg/ml. Five samples namely *A. karroo* (ethyl acetate extract), *P ecklonii* (ethyl acetate extract), *Senecio inonartus* (ethyl acetate extract), *S. inonartus* (chloroform extract) and *Aloe arborescens* (ethyl acetate extract) showed good MIC against *L. monocytogenes* and a MBC range from 1.0 to 12.5 mg/ml. The two plants, *A. karroo* and *P. ecklonii* were further selected for the isolation of the active compound(s).

Column chromatographic purification of ethyl acetate extracts of the leaves of *A. karroo* led to the isolation of three known pure compounds namely β-sitosterol, epigallocatechin and epicatechin. The MICs of the β-sitosterol and epigallocatechin that were isolated from *A. karrroo* were found to be 31.25 µg/ml and 62.5 µg/ml respectively against *L. monocytogenes.* The confocal scanning laser microscopy (CSLM) showed that the biomass of the listerial biofilms were reduced when the isolated compounds were added and slightly

reduced when the crude extract was added. The aggregation of cells which were exposed to β-sitosterol and epigallocatechin was reduced from 25 µm as observed in untreated cells to < 10 µm in length. Therefore as one of the local South African plants identified in the present study, the pure compounds isolated from *A. karroo,* could be used as a potential natural alternative for eliminating *L. monocytogenes* biofilms from food processing surfaces. This could help in combating the problem of food contamination and food poisoning caused by the pathogen. It could also help in preparing antibiofilm agents that are cost effective and easily accessible to the public. *A. karroo* should be further be explored in this regard. The present study reports for the first time the isolation of the three compounds, β-sitosterol, and epicatechin and epigallocatechin from *A. karroo*.

Bioassay-guided fractionation of the *P. ecklonii* ethyl acetate extract led to the isolation of two known compounds, parvifloron D and parvifloron F. Parvifloron D and F exhibited a minimum inhibitory concentration (MIC) of 15.6 and 31.25 µg/ml respectively against *L. monocytogenes.* The MICs of parvifloron D and F against a drug-sensitive strain of *Mycobacterium tuberculosis* were found to be 190 and 95 µg/ml respectively. The ethyl acetate extract of *P. ecklonii* and its isolated compounds were tested for their activity on tyrosinase inhibition. The concentration of plant extract at which half the tyrosinase activity was inhibited (IC_{50}) was found to be 61.73 ± 2.69 µg/ml. The antibacterial activity of the extract of *P. ecklonii* and its isolated compounds correlates with the traditional use of the plant for various ailments such as stomach-aches, diarrhoea and skin diseases. This is the first report on the bioactivity of an extract of *P. ecklonii* and its two compounds.

The antibacterial activity of the extracts of *A. karroo*, *P. ecklonii* and their isolated compounds correlates with the traditional use of these plants for symptoms associated with listeriosis.

CHAPTER 1

INTRODUCTION

Chapter 1

INTRODUCTION

1.1 Background

1.1.1 Historical evidence of the traditional use of medicinal plants

People in all continents have been using infusions of plant extracts for various ailments for centuries. Over decades, traditional medicine has significantly increased as global importance with profound effect in both health and international trade. In both Old and New worlds some of the archaeological records showed that medicinal plants have been used for centuries as remedies for human diseases because of their therapeutic value (Nostro *et al*., 2000). Civilisations of the ancient Chinese, Indians, and North Africans furnished written proof of man's resourcefulness in utilising plants for the treatment of a wide variety of ailments (Phillipson, 2001).

Egyptian pharaohs sent scouts far and wide searching for medicinal plants (Schultes & Von Reis, 1995). In ancient Egypt, garlic was used as part of early diet and it was fed to people involved in heavy labour such as building of the pyramids. Garlic was also used for the treatment of abnormal growths such as abscesses. In ancient Greece, garlic was fed to the soldiers to give them courage during war and and was also given to the athletes before they competed the Olympic games. Garlic was also used to protect the skin against poisons and toxins. Hippocrates, widely regarded as the father of Medicine advocated the use of garlic for various ailments. In ancient Rome, Japan and China, garlic was used for digestion, respiratory ailments,

arthritis, convulsions, animal bites, alleviate depression and for male potency (Rivlin, 2001).

In the middle of nineteen century, not less than 80% of medicines were developed from plants. Aspirin, atropia, artemisinin, atropine, taxol, morphine, reserpine, pilocarpine, quinine, digoxin, ephedrine and quinidine are some of the examples of plant-derived drugs that were initially discovered through the study of traditional remedies and folk knowledge of indigenous people. From the opium poppy (*Paper somniferum*), morphine was isolated and is one of the early molecules that entered into conventional medicine as the best painkiller available to humans. Reserpine isolated from the roots of *Rauwolfia serpentine* made in roads in modern Western medicine in 1949 for its ability to treat hypertension. Later it was established through clinical research that interest in the product gradually diminished when safer antihypertensive drugs were available after the unfavourable side effects of reserpine were determined. The adverse effects were depression and parkinsonism (Gilani and Atta-ur-Rahman, 2005). An active compound from Willow bark, aspirin is considered as one of the most effective analgesic, antipyretic, and anti-inflammatory agents that is usually utilised in modern medicine. Aspirin has been found recently to have antiplatelet/anticoagulant properties. The raw ingredients of a common aspirin tablet have been used as painkiller long before the tablet manufacturing machinery was invented (Gilani and Atta-ur-Rahman, 2005).

Taxol obtained from the bark of *Taxus brevifolia* was reported to have anticancer effect. Although the clinical trials did take place in the early 1980's it was until the 1990's that the semisynthetic derivative of taxol, taxotere were shown to be clinically active against breast and ovarian cancers. From *Artemisia annua*, the compound artemisinin has been shown during clinical trials that it is antimalarial and can be used to treat infections of multidrug resistant strains of *Plasmodium falciparum* (Phillipson, 2001).

Example of the drug developed from medicinal plant that failed in the clinical trial is bruceantin. Bruceantin was first isolated from a tree, *Brucea antidysenterica* (Simaroubaceae), used in Ethiopia for the treatment of cancer exhibited activity in animal models bearing tumours, but no object responses were observed in clinical trials. Further development for the drug was terminated (Gragg and Newman, 2004). The compounds, 'lapachol' isolated from the species *Tabebuia rosea* and *T. serratifolia* showed significant in vivo anti-tumour activity in some early mouse models and was advanced to clinical trials in the 1970s, but they were terminated due to high unacceptable levels of toxicity (Gragg and Newman, 2004).

Europeans discovered a tree called 'Quin-Quin' on the Eastern slope of Andes Mountains in the early 1600s. The Indians used the bark of quin-quin to cure malaria (Kaufman, 1989). Historians have produced evidence that show that people in the past used plants as medicine. From *Cinchona* bark, quinine was used to treat the symptoms of malaria long before the disease was identified (Phillpson, 2001).

The information on medicinal use of plants in Brazil was recorded in the book '*Historia Naturales Brasiliae*' in 1648 by the physician Guilherme Piso (Schultes & von Reis, 1995). In Mexico and Guatemala, many engravings narrate the use of different medicinal or hallucinogenic plants by local people (Schultes & von Reis, 1995). Use of medicinal plants has been stated for centuries as an aphrodisiac, as a tonic to increase mental and physical efficiency, to combat stress and as a cure for many human illnesses (Kaufman, 1989). In Iraq plant materials of a number of species have been found to have some horticultural use but a significant number of plants are also being used in local medicine as well. Chinese ethnopharmacological knowledge dates back several thousand years and some written records date from the beginning of the Christian era (Schultes & von Reis, 1995).

In Latin America 71% of the people in Chile and 40% in Columbia use traditional

medicinal plants. In China 40% of the health care is traditional medicine. In the United Kingdom 40% of allopathic practitioners offer the use of traditional medicine. Forty two percent of the population in the US, 70% in Canada, 48% in Australia, 38% in Belgium have at least used traditional medicine once at some stage (Bussmann and Sharon, 2006).

Many countries in Africa, Asia and Latin America use traditional medicine to help meet some of their primary health care. In industrialized countries, adaptations of traditional medicine are termed "complementary" or "alternative". In China, traditional herb medicine preparations account for 30 % - 50% of the total medical consumption these days. In Ghana, Mali, Nigeria and Zambia, 60% of children with high fever resulting from malaria is treated first with an herbal medicine at home (Kassaye *et al*., 2006).

1.1.2 The use of traditional medicine in South Africa

In Africa a move has begun to record medicinal plants, evaluate them and make valuable products (Neuwinger, 2000). A text has been compiled (Ross, 2003) describing the traditional use, chemical constituents, pharmacological activities and clinical trials of the commonly used medicinal plants found in Africa. Various African communities still use traditional remedies for primary health care (Louw *et al*., 2002). It is through the development of traditional medicine that the therapeutic effect of drugs has been revealed (Hikino, 1991). Despite the important contribution that medicinal plants make to primary health care, Western and traditional medicine systems seldom work together (Taylor & Van Staden, 2001).

In developing world, particularly in Africa up to 80% of the population uses traditional medicine as part of primary health care system (Bussmann and Sharon, 2006). About 4000 species of plants are used as traditional remedies for ailments in southern Africa (Van Wyk

and Gericke, 2000). In South Africa a large section of utilised medicines are still derived from plants and their extracts. Plants and their extracts are sold both in the informal and the commercial sector of the economy. Up to 60% of the South African population consult one of an estimated 200 000 traditional healers in preference to or in addition to Western medical doctors (Van Wyk *et al*., 1997; Taylor and Van Staden, 2001). An estimation of 27 million South Africans use traditional herbal medicine from more than 1020 plant species (Stafford *et al.*, 2005). Although traditional medicine is firmly rooted in the past, it is a dynamic and adaptive indigenous system of medicine.

South African medicinal plants are used for a wide variety of ailments such as diarrhoea, headaches, heart problems, inflammation, prevention of abortion, etc. South Africa's contribution to the world's medicines from plants are, just to name a few, 'Buchu' *(Agathosma betulina*) for the treatment of kidney and urinary tract, stomach problems, rheumatism and wounds, *Aloe ferox* for the treatment of eczema, arthritis, conjunctivitis, hypertension, stress and as a laxative, and 'Devil's claw' (*Harpagophytum procumbens*) for the treatment of rheumatism and arthritis (Van Wyk *et al.,* 1997; Van Wyk and Gericke, 2000). *H. procumbens* has been reported for the treatment in reducing the pain associated with osteoarthritis and other related chronic conditions (McGregor *et al*., 2005). *H. procumbens* and *Sutherlandia frutescens* have been reported to be used in South Africa for the management of pain and inflammation (Kundu *et al*., 2005). The phytochemical screening of *H. procumbens* was found to have iridoid glycosides, acetylated glycosides and terpenoids. The *S. frutescens* has been reported to contain arginine, γ-aminobutyric acid and pinitol (Kundu *et al*., 2005). *S. frutescens* has been reported to have antibacterial activity against *Staphylococcus aureus*, *Enterococcus faecalis* and *Escherichia coli.* The medicinal use of *S. frutescens* for staphylococcal infections, when formulated in an oily base seems to have a rational basis (Katerere and Eloff, 2005). One plant that is indigenous to

South Africa and Namibia that has been shown to have a commercial spin off is *Hoodia* in particular the *H. gordonii species. H. gordonii* belongs to the *Apocynaceae* family. *H. gordonii* has been found to have an appetite suppressant properties (Van Heerden, 2008; Lee and Balick, 2007; Van Heerden *et al*., 2007). *Hoodia* is being used by San people of South Africa as an appetite suppressant. The species has been commercialised as a weight loss product and therefore, it has been widely marketed (Vermaak *et al*., 2010(a)). *H. gordonii* has been marketed as a functional food and products in dosage form that include tablets, capsules, powders, sprays, tea, fruit and chocolate bars (Vermaak *et al*., 2010(b)). Another species of *Hoodia*, *H. pilifera* has also been found to have appetite suppressing activity on rats. It is not surprising that the *Hoodia* species has been patented (Van Heerden *et al*., 2007; Moyer-Henry 2008). The Council for Scientific Research Council (CSIR) patented the compound responsible for suppression from *H. gordonii* and named it P57. Initially CSIR did not give any credit to San people, the owners of indigenous knowledge about *Hoodia* who accused CSIR and its partners of biopiracy. The South African government intervened by offering cash settlement to San people for using their indigenous knowledge. The San people from four countries South Africa, Botswana, Namibia and Angola were offered payment of at least $30 000 during clinical testing of *Hoodia* and 6% of the royalties received on the market (Moyer-Henry, 2008). This clearly shows that the bioactive compounds from plants do play a role in discovering remedies for various ailments.

The following South African plants, *Artemisia afra*, *Pteronia incana* and *Rosmarinus officinalis* were tested against *L. monocytogenes* and other pathogens. The three plants were found to have antilisterial activity (Mangena and Muyina, 1999; Sandasi *et al*., 2010). Another South African medicinal plant, *Agathosma betulina* has been reported to have antilisterial activity (Molla and Viljoen, 2008). *Echinacea angustifolia*, *Thymus vulgaris* and *Mentha*

piperita are some of the South African plants that have been reported to have antilisterial activity. The plants were also found to have inhibitory effect against the formation and development of the listerial biofilm (Sandasi *et al*., 2010).

Listeriosis is reported mainly from industrialized countries with few or no reports from Africa, Asia and South America (Rocourt *et al*., 1990). In South Africa there is a dearth of literature on the epidemiology of the microorganisms. South Africa has one of the highest rates of HIV infection in Africa and the immunocompromised are susceptible to listerial infection. The last official report of listeriosis from South Africa was in 1978 (Jacobs *et al*., 1978). There is limited research on the South African plants that have antilisterial activity. Most of the research is on plants that have antimycobacterium, antimalaria, anti-oxidant and antiHIV activities. Due to the side effects of existing antilisterial drugs it was decided to explore the potential of South African plants for listerial infections in the present study.

1.2 Listeriosis

1.2.1 Epidemiology

Listeria monocytogenes is a food-borne pathogen of public health concern (Schmid *et al*., 2009) which causes serious diseases such as encephalitis sepsis and meningitis, endocarditis, etc in humans. A pregnant woman infected with these bacteria may suffer from flu-like febrile illness and depending on the stage of pregnancy the foetus may be either stillborn (abortion) or born with signs of infection (Goldenberg and Thompson, 2003; de Souza *et al*., 2008). *L. monocytogenes* is widespread in nature and because of its ubiquity, the pathogen is frequently isolated from foodstuff and food related environments (Amalaradjou *et al*., 2009). *L. monocytogenes* is pathogenic not only to humans but also to animals (Hof, 2003(a)). *L.*

monocytogenes is found widely in a variety of ready-to-eat foodstuff (Saunders *et al*., 2005, Schmid *et al*., 2009). People most prone to the disease are pregnant women, newborns, the elderly, and those with Human immuno virus (HIV) and immunocompromised (DiMaio, 2000; Békondi *et al*., 2006). Listeriosis is a severe human infection, characterised by gastro-enteritis, meningitis, encephalitis, septicaemia, spontaneous abortions and deaths (Guzman *et al*., 1995; Goldenberg and Thompson, 2003; de Souza, 2008). Listeriosis is mainly reported from industrialised nations with few or no report from Africa, Asia and South America (Racourt *et al*., 2000). In most industrialised countries listeriosis has been made a reportable disease and these countries have implemented active surveillance of food and food processing plants (Pagotto, *et al*., 2006). Table 1.1 shows gastrointestinal listeriosis outbreak from 1993 to 2001 reported in the industrialised nations.

Table 1.1. Gastrointestinal listeriosis outbreaks, 1993-2001 (Table adapted from Swaminathan and Gerner-Smidt, 2007)

Year	**Location**	**Number of cases**	**Implicated source**
1993	Northern Italy	18	Rice salad
1994	Illinois, USA	44	Chocolate milk
1997	Northern Italy	1566	Cold corn and tuna salad
1998	Finland	N/A	Cold-smoked fish
2000	New Zealand	32	Ready–to-eat meat
2001	California, USA	16	Delicatessen turkey ready-to-eat meat
2001	Sweden	48	Raw milk cheese
2001	Japan	38	Cheese

N/A: number of cases not given.

Symptoms of listerial meningitis usually begins with mild flu, followed by a sudden pulsating fever, pain, stiffness of the neck and back, nausea, vomiting, lethargy, flu, headache, and inflammation (Nester *et al*., 2001; Prescott *et al*., 2005).

Cases of listeriosis have been reported in Canada), the United States, Japan, Germany, Australia, South Africa and in other parts of the world. *L. monocytogenes* is a zoonotic foodborne pathogen that is responsible for 28% of food related deaths in the United States annually (Borucki *et al*., 2004; Amalaradjou *et al.*, 2009). When an outbreak of listeriosis cropped up in California in 1985 (Table1.2), almost every case was traced back to a fresh, soft cheese made with contaminated milk that had not been properly pasteurised. Forty-eight deaths (Table 1.2) were reported of which 30 were among foetuses and newborns (Talaro and Talaro, 1993). Mortality cases of listeriosis in 2 500 cases per year in the US has mortality of approximately 20% (Tominga, 2006). Because of its widespread in nature, *L. monocytogenes* has been frequently isolated from foods and food processing environments. *L. monocytogenes* has been found to be able to adhere all food contact surfaces such as glass, stainless steel and rubber (Amalaradjou *et al*., 2009). The organism is a challenge to food production industry as well as food related environments (Schmid *et al*., 2009). Forty-two cases of listeriosis from human isolates were reported in Maryland and California in 2000 and 2001, out of the 4500 isolates from cases that had occurred throughout the United States (Gray *et al*., 2004).

A listeriosis epidemic in newborns was recorded in Germany in 1949. In eighty-five newborns or stillborn infants, granulomas were detected histopathologically in various organs such as the spleen, brain, lung, liver and skin (Hof, 2003(a)). During the same year, Holland reported 4.3 cases per million inhabitants per year. England and Wales reported 2.6 cases per million inhabitants per year from 2001 to 2004.

Table 1.2 Outbreaks of invasive listeriosis, 1981-2003 (Adapted from Swaminatham and Gerner-Smidt, 2007)

Year	Location	No. of cases	Perinatal cases	No of deaths	Source/implicated vehicle
1981	Nova Scotia, Canada	41	34	18	Coleslaw
1983	Massachusetts, USA	49	7	14	Pasteurized milk
1985	California, USA	142	94	48	Mexican-style cheese
1983-1987	Switzerland	122	65	34	Vacherin Mont d'Or cheese
1987-1989	United Kingdom	366	?	?	Paté
1989-1990	Denmark	26	3	7	Blue mold cheese
1992	France	279	0	85	Pork tongue in jelly
1993	France	38	31	10	Rillets
1998-1999	Multiple states, USA	108	?	14	Hot dogs
1999	Finland	25	0	6	Butter
1999-2000	France	32	9	10	Pork tongue in aspic
2000	Multiple states, USA	30	8	7	Delicatessen turkey ready-to-eat meats
2000	North Carolina, USA	13	11	5	Home-made Mexican – style cheese
2002	Multiple states, USA	54	12	8	Delicatessen turkey
2002	Quebec, Canada	17	3	0	Cheese made from raw milk

? Number not known

Fifty-seven cases of listeriosis occurred in Switzerland during an outbreak, which was caused by the consumption of soft cheese (Bula *et al*., 1995). The mortality was reported to be 32% from the 57 cases. In Japan a nationwide surveillance study of listeriosis was performed and from the data collected between 1980 and 2002, 95 cases were identified (OzFoodNet Working Group, 2003).

In Johannesburg (South Africa) during August 1977 to April 1978, 14 patients were reported to have systematic infections due to *L. monocytogenes* (Jacobs *et al*., 1978). Out of the 14 patients, nine were neonates who had septicaemia and meningitis. The mortality rate was 43% (Jacobs *et al*., 1978). It has been found that 17% of patients admitted to hospital with prosthetic endocarditis (caused by *L. monocytogenes*) die in hospital (Miguel-Yanes *et al*., 2004).

1.2.2 Listeriosis in animals

L. monocytogenes pathogen does not only affect humans but also the animals. According to Jemmi & Stephan (2006) infection in both animals and humans occurs in the following manner, namely, an entry of the pathogen into the host, lysis of phagosomal vacuole, multiplication in the cytosol and finally the direct cell-to cell spread using actin-based motility. Cattle and other ruminants (Figure 1.1) can be infected with *L. monocytogenes* by consuming contaminated plant materials, soil or silage (Mantovani & Russel, 2003). In ruminants, *L. monocytogenes*, primarily causes encephalitis and uterine infections. These infections are characterised by late-term abortions or septicaemia in neonates (Nightingale *et al*., 2004). It is clear from literature (Loeb, 2004; Miyashita *et al*., 2004; Nightingale *et al*., 2004; Evans *et al*., 2004) that listeriosis does affect the animals in the same way as it affects humans. Septicaemia and abortions in a housed flock of sheep have been reported (Low & Renton, 1985). An outbreak

of listeriosis in horses, cattle and other ruminants have been reported (Vandegraaf *et al*., 1981; du Toit, 1977; Loeb, 2004*).* In Germany, Bavarian cattle clinically suspected of bovine spongiform encephalitis were diagnosed with listeriosis (Miyashita *et al*., 2004). Du Toit (1977) reported an outbreak of listeriosis in the Western Cape province of South Africa. Abortion in sheep caused listeric-infection has been reported (Dennis, 1975). The cause of listeriosis in Gippland, Victoria in 1978 was confirmed by the histology of the brain or culture of *L. monocytogenes* from sheep on 21 farms (Vandergraaf *et al.,* 1981). The fatality rate from the affected flocks (0. 2 to 8.0 %) was almost 100 %. An analysis of 42 ruminants with suspected meningo-encephalitis caused by *Listeria* was reported in the Netherlands (Loeb, 2004). Listeriosis may be the cause of eye infections in ruminants and horses (Evans *et al*., 2004).

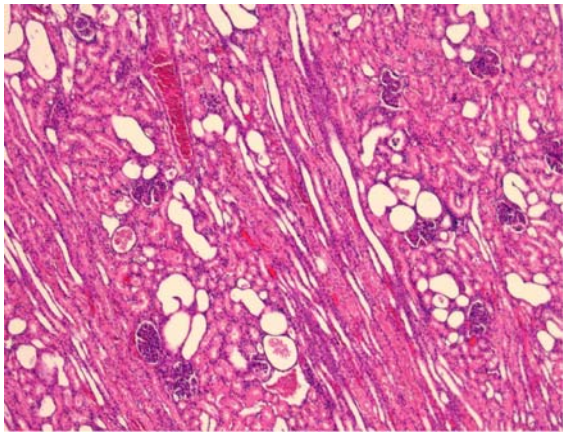

Figure 1.1 Characteristic lesions of acute renal tubular necrosis including renal tubular dilation and flattening of the renal tubular epithelium in sheep caused by *L. monocytogenes* (magnification x 40) - photo William Byrne (Regional Veterinary Laboratories, 2006**)**

1.2.3 Causative agent of listeriosis – *Listeria monocytogenes*

Listeria belonging to the family *Listeriaceae* contains short rods that are aerobic or or facultative, catalase positive, and motile (Prescott *et al*., 2005). The genus comprises six species,

two pathogenic species *L. monocytogens* and *L. ivanovii*, and four non-pathogenic *L. welshimeri*, *L. grayi*, *L. innocua* and *L. seeligeri*. *L. monocytogenes* was first discovered in 1924 by E.G.D Murray when he isolated the gram positive rods from the blood of rabbits in the laboratory. It was called *Bacterium monocytogenes,* the genus name was then changed to *Listeria* by J.H.H. Pirie in 1940 after it was isolated from humans (Hof, 2003 (a)). *L. monocytogenes* is a facultative anaerobic motile non-spore forming species (Prescott *et al.,* 2005). Factors that affecting the pathogenicity of *L. monocytogenes* its capacity for intracellular growth, iron compounds, catalase and superoxide dismutase, surface components, hemolysins – have been proposed over the years, indicating that its virulence is caused by a number of factors (Farber and Peterkin, 1991). It is a ubiquitous opportunistic pathogen causing fatal infections known as listeriosis (Poros-Gluchowska & Markiewicz, 1988; Dussurget *et al*., 2004).

1.2.4 Drug resistance of listeriosis

Listeriosis is treated with a series of different antibiotics (Hof, 2003 (b)). Drugs that are available to treat listeriosis in humans are ampicillin, trimethoprim sulfamethoxazole, erythromycin, vancomycin, fluroquinolones, etc. (Becq-Giraudon and Breux, 1987; Temple and Nahata, 2001; Benes *et al*., 2002; Poros-Gluchowska and Markiewcz, 2003; Gunter and Philipson, 1988; Friedrich *et al.,* 1990).

The antibiotic, 'Meropenem' has been found to be effective in the treatment of meningitis caused by *L. monocytogenes* in animals (Nairn *et al*., 1995). Meropenem is able to penetrate into the cerebrospinal fluid (CSF) to produce effective reduction in a number of pathogens (Nairn *et al.,* 1995). The drugs used for the treatment of listeriosis in animals are coumermycin Hof, 1991) and levofloxacin (Nichterlein *et al.,* 1998(a)). Levofloxacin has been found to be

(effective in the treatment of infections caused by facultative intracellular Gram-positive such as *L. monocytogenes* (Nichterlein *et al*., 1998(a)).

L. monoctogenes has been found to be resistant to some antibiotics (Nichterlein *et al.,* 1998(b); Temple & Nahata, 2000; Hof, 2003 (b); Friedrich *et al*., 1990). In most cases (Cone *et al*., 2003) it has been found that listeriosis treatment needs multidrug therapy. Therefore the combination of two or more antibiotics is needed for treatment (Cone *et al*., 2003; Poros-Gluchowska and Markiewicz, 2003; Temple & Nahata, 2000; Nichterlein *et al*., 1998 (b); Rossi *et al*., 2001; Hof, 2003 (b); Friedrich *et al*., 1990). Therapy with high dose of ampicillin in combination with gentamicin was the preferred treatment in humans (Cone *et al.,* 2003). Most antibiotics are not bactericidal for *L. monocytogenes.* The combination of various drugs may exert a synergistic effect against *L. monocytogenes* (Hof, 2003 (b)). It has been reported that erythromycin is inefficient against *L. monocytogenes* in multi-drug resistant cells. Erythromycin was unable to curb the growth of *L. monocytogenes* in multi-drug resistant human carcinoma cell lines (KBV-1 MDR cells) but restricted the growth of the bacteria in non-resistant human epidermoid carcinoma cell lines (KB3-1 cells) (Nichterlein *et al*., 1995).

Certain host cells may have gained the property of eradicating some bacteria, for example macrolides (family of antibiotics used to treat a wide range of bacterial infections) from intracellular spaces, which might elucidate therapeutic failures of antibiotic therapy in spite of low MICs (Hof *et al*., 1997). The high cost involved in the manufacturing of the synthetic drugs has also made the available drugs used for the treatment of listeriosis expensive. The combination of trimethoprim/sulfamethoxazole is the preferred treatment for listeriosis (Gleckman & Borrego, 1997; Poros-Gluchowska & Markiewcz, 2003). Ampicillin, amoxicillin vancomycin, erythromycin, are also used for the treatment of listeriosis. The side effects of these drugs are quite alarming. Trimethoprim/sulfamethoxazole increases the risk of

hypoglycaemia, bone marrow suppression and increases the anticoagulant effect in patients. Ampicillin increases the frequency of rash, vancomycin results in hypersensitivity reactions (red-neck or red-man syndrome) and amoxicillin causes diarrhoea (Gleckman & Borrego, 1997). In one study it was reported that drug related toxicity was one of the most common causes of death for hospitalized patients (Gleckman & Czachor, 2000). Finding the most effective novel drugs from plants against *L. monocytogenes* could reduce the risk of multidrug resistant species and reduce the treatment costs.

1.3 Scope of the thesis

1.3.1 Antilisterial activity of plant extracts

Medicinal plants such as *Clivia miniata, Artemisia afra, Aloe arborescens, Tulbaghia violacea, Heteromorpha arborescens*, etc are used by South Africans to treat listeriosis related symptoms such as fever, flu, headache, inflammation, heart condition, etc. (Van Wyk *et al*., 1997; Van Wyk & Gericke, 2000). The high cost of synthetic drugs and the problem of multidrug resistance have necessitated the need to explore the potential of South African medicinal plants for antilisterial activity. The extracts from plants are inexpensive and accessible. The antilisterial activity of local medicinal plants that have been used to treat listeriosis symptoms was investigated for this study.

1.3.2 Susceptibility testing (to the plant extracts) of *L. monocytogenes*

The disc diffusion method was used to test antibacterial activity against *L. monocytogenes* in order to select the best extract for further tests as described by Alzoreky & Nakahara (2003).

The disc method is usually used to exhibit growth inhibition of microorganism by plant extracts (Masika and Afolayan, 1998; Rabe and Van Staden, 1998; Pretorius *et al*., 2003). Sterile filter paper impregnated with plant extracts were placed on petri dishes. Bacterial culture was spread on Petri dishes and incubated for 24 hours. Zone of bacterial inhibition was determined. Selected exracts were investigated to determine their minimum inhibitory concentrations (MIC) against *L. monocytogenes* using the microtitre dilution method (Eloff, 1998). In the liquid medium there is both contact of the extract and microorganism hence this is more sensitive and accurate than the disc diffusion. The *p*-iodonitrotetrazolium violet (INT) was used to determine the viability of bacteria. The microtitre method or microboth dilution method by Eloff (1998) has been used extensively by researchers in the determination of the MICs (Stafford *et al*., 2005; McGaw *et al*., 2007; Buwa and Afolayan, 2009; Amoo *et al*., 2009; Mulaudzi *et al*., 2009).

1.3.3 Cytotoxicity assay of plant extracts

Cytotoxicity evaluation of the plant extracts and their active principles are required for their effective therapeutic uses. Cytotoxicity tests are essential to ascertain the intrinsic ability of the extract or compound to cause harm to the cells or cell death as a result of damage to cellular functions (Bouaziz *et al*., 2006). Cytotoxicity tests are also necessary in the development of the potential drug as these tests provides crucial information on *in vitro* testing on parameters such as genotoxicity or programmed cell death (Bouaziz *et al*., 2006).

After establishing the antilisterial activity of 13 plant extracts against *L. monocytogenes*, the next step was to isolate the active compound(s) from one of the most active and least toxic of the plants. Cytotoxicity assay of plant samples were carried out on Vero cell lines with the intention to select a plant for the isolation of the active compound(s) with good antilisterial activity and

low toxicity.

1.3.4 Isolation, purification and identification of the active compound(s) from *Acacia karroo*

The isolation of natural products that have biological activity toward organisms other than the source has several advantages. The first advantage is that pure bioactive compounds can be administered in reproducible, accurate doses, with obvious benefits from an experimental or therapeutic point of view. Secondly, it can lead to the development of analytic assays for particular compounds or classes of compounds. This is necessary, for example in the screening of plants for potential toxicity and for quality control of food for human and animal consumption. Thirdly, it permits the structural determination of bioactive compounds that may enable the production of synthentic material, incorporation of structural modifications, and a rationalization of mechanisms of action. This, in turn, will lead to reduction in the dependency of plants as sources of bioactive compounds and will enable investigations of structure/activity relationships, facilitating the development of new compounds with similar or more desirable bioactivities.

Out of the thirteen plants screened for activity and cytotoxicity it was found that *Acacia karroo* and *Plectranthus ecklonii* exhibited the highest antilisterial activity as compared to the other plants investigated. Our objective was to isolate the bioactive compound(s) from *A. karroo* and *P. ecklonii* and determine the minimum inhibitory concentration (MIC) of the isolated compounds against *L. mononcytogenes*. Through the bioassay guided fractionation of the ethyl acetate extracts of *A. karroo* two bioactive compounds were isolated and identified. The MICs of the isolated compounds were determined against *L. monocytogenes.*

Bacterial biofilms are more resistant to the action of antimicrobial and disinfectant agents (García-Almendárez *et al*., 2007). Briefly, a standardized overnight culture was allowed to develop a biofilm on glass slides that had been previously coated with hundred microlitre of tryptone soya broth (TSB) to provide nutrients for adhering bacteria (Chae and Schraft, 2000). The viability and distribution of listerial cells exposed to crude extracts and pure compounds were investigated using the method described by Kives *et al*. (2005). Biofilms of *Listeria* were prepared by growing *L. monocytogenes* cells on sterile cover slips. The aggregation of the *Listeria* biofilm on exposure to the plant extracts and / or the purified compounds was investigated.

1.3.5 Isolation, purification and identification of the active compound(s) from *Plectranthus ecklonii*

Plectranthus ecklonii Benth. is traditionally used in South Africa for treating stomach-aches, nausea, vomiting and meningitis, all symptoms associated with listeriosis infection. Leaves of the plants are also used for respiratory problems, chest complaints and coughs (TB-related problems) (Lukhoba *et al*., 2003). The pure compounds as well as the crude extract of *P. ecklonii* were tested for activity against *L. monocytogenes*, *Staphylacoccus aureus, Escherichia coli, Mycobacterium tuberculosis,* and *M. smegmatis*. The minimum inhibitory concentration (MIC) and minimum bactericidal activity (MBC) of *P. ecklonii* were determined. Melanin is a key pigment responsible for skin and / or hair colour. Tyrosinase is an enzyme that catalyses the production of melanin. The inhibition of tyrosinase has an effect on the production of melanin. The skin problem such as albinism is a lack of tyrosinase. The antityrosinase activity of the extract of *P. ecklonii* and its isolated compounds were also investigated

1.4 References

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Chapter 2

BACKGROUND INFORMATION ON THE SELECTED PLANTS

Chapter 2

BACKGROUND INFORMATION ON THE SELECTED PLANTS

Abstract

South African medicinal plants were selected based on information of their traditional medicinal use (traditional healers) in treating symptoms of listeriosis (diseases caused by *L. monocytogenes* infection) and also based on a literature review. The plants selected were *Aloe arborescences*, *Acacia karroo*, *Artmesia afra*, *Clivia miniata*, *Eucomis autumnalis*, *Datura stranomium*, *Drimia altissima*, *Gomphocarpus fruticosus*, *Heteromorpha arborscens*, *Tulbaghia violaceae*, *Senecio inonartus*, *Ziziphus mucronata* and *Plectranthus ecklonii*. The medicinal use, phytochemistry and distribution of each plant are described.

2.1 Introduction

South Africa still utilises a large selection of medicines that are derived from plants and their extracts (Van Wyk *et al*., 1997; Taylor & Van Staden, 2001). In the past and the present herbal medicine has become a topic of augmented global importance, having strong influence on both world health and international trade (Stafford *et al.*, 2005).

2.2 Plants selected for the present study

Thirteen medicinal plants were collected from Gauteng, northern and eastern Free State, South Africa. Different parts of the plants for example, leaves, stem, bark, roots were collected. The symptoms of listeriosis is gastro-enteritis, severe headaches, pulsating fever, nausea, vomiting, stiffness of the neck and back, septicaemia, and abortion. Traditional healers were asked for their treatment they use when patients complain and show these symptoms. The questionnaire (Appendix 3, page 134) was used to get information from the traditional healers. The healers mentioned vernacular name. An effort was made to go with the healers to find out the plants they use. Herbarium specimens were made which assisted to find out the scientific names. The plant species collected were based on information received from experienced traditional healers and elderly indigenous people who are experts on traditional medicine. The selection of these plants were also based on information culled from literature. The samples were taken to the HGWJ Schweickerdt herbarium of the University of Pretoria for identification. The herbarium specimens are preserved in the aforesaid herbarium (Table 2.1).

Table 2.1 Voucher specimen numbers of the selected plants used for the present study.

Plant species	**Voucher Specimen number**	**Plant part used**
Acacia karroo Hayne	MN15	Leaves
Aloe arborescens Mill.var. *natalensis* Berger	MN 5	Leaves
Artemisia afra Jacq.	MN 7	Leaves
Clivia miniata Reg.	MN 3	Whole plant
Datura stramonium L.	MN 8	Leaves
Drimia altissima (L.f.) Ker Gawl	MN 14	Roots Leaves
Eucomis autumnalis (Mill.) Chitt.	MN 11	Bulb
Gomphocarpus fruticosus (L) W.T. Aiton	MN 1	Leaves
Heteromorpha arborescens (Spreng) Cham. &. Schltdl.	MN 4	Stem Leaves
Plectranthus ecklonii Benth.	PRU 96396	Leaves
Senecio inonartus DC	MN 9	Leaves Stem
Tulbaghia violaceae Harv.	MN 2	Leaves
Ziziphus mucronata Wild.	MN 10	Leaves

The detailed descriptions of the selected plants for the present study are as follows:

2.2.1 *Acacia karroo* Hayne

A.karroo is a member of the *Fabacea*e family. It is commonly known as sweet thorn. In the different South African languages it is known by different names in Zulu it is known as

"umunga" and in Xhosa "umnga", "mookana" in Pedi (Northern Sotho). Its trunk is made up of "rough bark, which is dark brown in colour. The branches have many pairs of white large spines. The small round yellow flowers give the plant its distinct character (Figure 2.1) *A. karroo* is used medicinally to treat diarrhoea, colds, dysentery, conjunctivitis and haemorrhage. The gum from *A. karroo* has been used medicinally as emollients and as pharmaceutical aids such as emulsifiers, stabilisers of suspensions and additives for solid formulations. The gum has also been used to treat mouth ulcers (van Wyk *et al*., 1997; van Wyk & Gericke 2000). In the previous study (Katerere & Eloff, 2004) *A. karroo* was found to have antibacterial activity against *Escherichia coli* and *Staphylococcus aureus*. Compounds which have been isolated from the heartwood (stem) of *A. karroo*, are (2'S,3'R)-3,10-dihydroxy-9-O-(6'-hydroxy-7'-O-methyl-2'-hydroxy-methyldihydrobenzofuran-3-yl)dibenz-[b,d]-pyran-6-one and its 10-O-methyl analogue; 8-O-methylepiprosopin-4β-ol; 8-methoxyfustin; and 7,8,3,4'-tetrahydroxy-3'-methoxyflavone (Malan & Swartz, 1995). The compound, 7,8,3,4'-tetrahydroxy-3'-methoxyflavone has been reported to have antioxidant and antimutagenic activities (Chung *et al*., 1999). The compound has also been reported to have antibacterial activity against the Gram postive bacteria such as *Bacillus cereus*, *Staphylococcus aureus*, *Enterococcus faecalis* and Gram negative bacterium, *Escherichia coli* (Wang *et al*., 1989; Teffo *et al*., 2010). The phenolic acids, cinnamic, caffeic, *p*-coumaric, ferulic acids and chlorogenic acids from fresh fruit and vegetables have been reported to have antilsterial activity (Wen *et al*. 2003). The phenolic acids were also found to be bactericidal against *L. monocytogenes*. These compounds have similar structures to other phenolics compounds that have been isolated from *A. karroo* such as acaciabiuronic acid (DNP, 2010). The leaves were used for the present study.

The Distribution of *A. karroo*

The genus *Acacia* is found in the dry regions of Africa (Katerere & Eloff, 2004; Dube *et al*., 2001), Australia, India and America (Arias *et al*., 2004). *A. karroo* is widely distributed in South Africa (Van Wyk *et al.*, 1997) and is found in all the nine provinces.

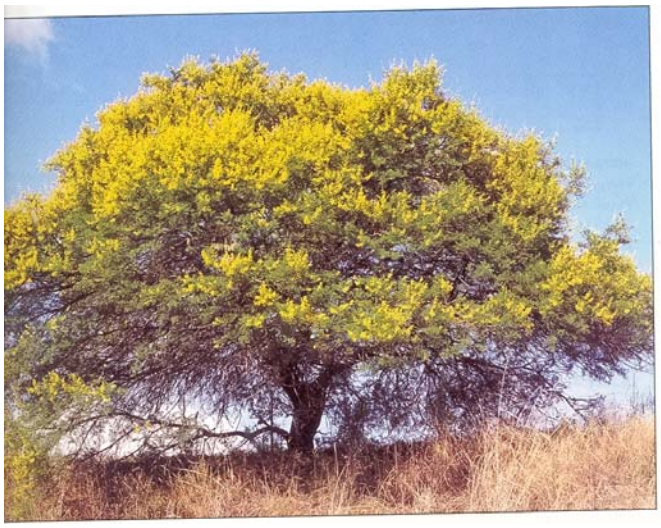

Figure 2.1 *Acacia karroo* a tree with the golden-yellow round flowers (Courtesy: Van Wyk *et al.*, 1997)

2.2.2 *Aloe arborescens* Mill. var. *natalensis* Berger

A. arborescens is a member of the family *Asphodelaceae.* It has long fleshy dull green leaves that have spines along the edges (Figure 2.2). The plant is medicinally used to treat wound and burns. *A. arborescens* has been reported to be used during pregnancy to ease labour (Grace *et al*., 2008). The plant has been reported to have antibacterial, anti-inflammatory anti-ulcer and anticancer activities (Van Wyk & Gericke, 2000; Jia *et al*., 2008). The compounds aloenin, aloenin B, 10-hydroxyaloin A, aloin A and B, and aloe-emodin have been isolated from the leaves of *A. arborescens*. Aloenin has been reported to have purgative and laxative activity (Park *et al*., 1998). *A. arborescens* has been found to be effective in wound healing. The species

has also been found to have antibacterial activity against *Stapyhlococcus aureus*, *Klebsiella pneumonia*, and *Escherichia coli* (Jia *et al*., 2008). The leaves were used for the present study.

Distribution of *A. arborescens*

A. arborescens is widely distributed in the eastern parts of South Africa especially in the Eastern Cape and some parts of Western Cape and KwaZulu -Natal. It is a popular garden plants found in most gardens in South Africa.

Figure 2.2 *Aloe arborescens* (Courtesy: Van Wyk *et al*., 1997)

2.2.3 *Artemisia afra* Jacq.

A. afra belongs to the family *Asteraceae*. It is known as African wormwood. In Zulu and Xhosa it is known as "umhlonyanae", and "lengana" in Sotho and Tswana. *A. afra* is a perennial shrub with greyish-green leaves (Figure 2.3), and yellow flowers. The leaves are medicinally used to treat coughs, fever, colds, influenza and blocked nasal passages. The plant has antimicrobial and anti-oxidative properties (Van Wyk *et al.*, 1997; Van Wyk & Gericke, 2000).

The methanol extract of *A. afra* has been found to have antibacterial activity against *S. aureus* and *Bacillus subtilis* (Rabe and Van Staden, 1997). The compounds isolated from *A. afra* which have been reported to have antimicrobial properties are: 1.8-cineole, α-thujone, β-thujone, camphor and borneol (Van Wyk *et al.*, 1997; Mangena and Muyima, 1999). These compounds have been isolated from the leaves. The 1.8-cineole, α-thujone, β-thujone isolated from *A. afra* have been found to have broad antibacterial activities against a number of bacteria such as *Salmonella enteretidis*, *S. typhi*, *S. newport*, *Bacillus subtilis*, *Micrococcus luteus*, etc. (Mangena and Muyima, 2009). Dichloromethane, water and ethanol extracts of *A. afra extracts* have been found to have antibacterial activity against *Bacillus cereus, E.coli, Klebsiella pneumonia, S. aureus* and *Mycobaterium aurum* respectively (Buwa and Afolayan, 2009). The leaves were used for this study.

Distribution of *A. afra*

A. afra is a widely distributed, common species in South Africa. It is distributed as far as north as tropical east Africa through to Ethiopia.

Figure 2.3 *Artemisia afra* (Courtesy: Van Wyk *et al*., 1997)

2.2.4 *Clivia miniata* Reg.

C. miniata belongs to the family *Amaryllidaceae.* It is a perennial tuberous rhizamatous plant that does not require exposure to sunlight to grow. It is commonly known as bush lily or orange lily. In Zulu it is known as "umayime." It usually bears orange flowers that develop from the same point of the stalk (Figure 2.4). The rhizome (underground stem) is medicinally used to treat fever and to relieve pain while the whole plant is used in childbirth. Roots and leaves are taken by South African women during pregnancy and childbirth (Louw *et al*., 2002). Compounds such as lycorine, clivacetine, clivonine, cliviasine and clividine have been isolated from *C. miniata*. Lycorine, clivonine and cliviasine have been isolated from the rhizome. Clivacetine and clividine have been isolated from the whole plant. Lycorine has been found to be a respiratory stimulant and has been reported to have antitumour, antiviral and antifungal activity (Van Wyk *et al*., 1997; Pieters and Vlietinck, 2005). The whole plant was used for this study.

Distribution of *C. miniata*

C. miniata is found on the eastern coastal line of South Africa, in the Eastern Cape and KwaZulu-Natal provinces (Van Wyk *et al*., 1997).

(a) (b)

Figure 2.4 *Clivia miniata* flowers (a) and rhizomes (b) (Courtesy: Van Wyk *et al*. 1997)

2.2.1.5 *Datura stramonium* L.

D. stramonium belongs to family *Solanaceae*. The plant is commonly known as the thorn apple. In Zulu it is known as "iloyi". It is has white or purplish tubular flowers and four–locular–fruit capsules which have brown, kidney shape seeds (Figure 2.5) (Van Wyk *et al*., 1997). The leaves of *D. stramonium* are medicinally used to relieve asthma, rheumatism, gout, boils, abscesses, sore throat, tonsillitis and respiratory difficulties. Methanol extract of *D. stramonium* has been found to be active against *Bacillus subtilis* (Rabe and Van Staden, 1997). In previous study (Mdee *et al*., 2009) *D. stramonium* was found to have antifungal activity against several tested fungi such as *Aspergillus niger, A. parasiticus*, etc. Tropane has been isolated from *Datura* species (Rao and Ravishankar, 2002). *D. stramonium* has been reported to contain lubumin, hydroxylubimin, germacrenediol and small amounts of capsidiol (Stoessl *et al*., 1976). Atropine and scopolamine are the two major compounds isolated from *D. stramonium*. The compounds have been isolated from the aerial parts of the plant. It has been reported that scopolamine has

analgesic, anti-inflammatory and antispasmodic activities.

Distribution of *D. stramonium*

D. stramonium is widely distributed in South Africa and is found in all the provinces.

Figure 2.5 *D. stramonium* (Courtesy: Van Wyk *et al*., 1997)

2.2.6 *Drimia altissima* (L.f.) Ker Gawl

D. altissima belongs to the family *Hyacinthaceae*. Species of *Drimia* have large underground bulbs, strap-shaped leaves (Figure 2.6) and long thin flowering stalks. The bulbs and leaves are medicinally used as expectorants (promoting secretion, liquefaction or expulsion of the sputum from the respiratory passages), emetics, diuretic and as a heart tonic, to treat fever, bladder and uterus disease (Van Wyk *et al*., 1997; Louw *et al*., 2002). The compound 3-*O*-α-L-Rhamnopyranoside has been isolated from *D. altissima* (DNP, 2010). Bufadienolides and cardioactive C-24 steroids have been isolated from *D. altissima* (Krenn & Kopp, 1998).

Bufadienolides have been isolated from the bulb. In the previous study *D. altissima* was tested for antibacterial activity against methicillin resistant *S. aureus* and was found to have low activity against the pathogen (Heyman *et al*, 2009). The roots and leaves were used for this study.

Distribution of *D. altissima*

D. altissima is widely distributed in South Africa particularly in the region of summer-rainfall region (Crouch *et al*., 2006).

(a) (b)

Figure 2.6 *Drimia altissima* (a) bulbs and (b) leaves

2.2.7 *Eucomis autumnalis* (Mill.) Chitt.

E. autumnalis belongs to the family *Hyacinthaceae*. This bulbous plant is commonly known as the pineapple flower. In Zulu and Xhosa it is known as “umathunga.” It consists of a number of small yellowish-green flowers that are attached to the centrally located stalk (Figure

2.7). The bulb is medicinally used for fever, stomach ache, colic, urinary diseases, diarrhoea, syphilis, easing of childbirth, etc. (Van Wyk, 2008). *E. autumnalis* has been reported to treat chest complaints, coughing and tuberculosis (McGaw et al., 2008). Methanol extract of *E. autumnalis* has been found to have antibacterial activity against *B. subtilis* (Rabe and Van Staden, 1997). *E. autumnalis* serves as an enema for low back pain, fever, colic and urinary diseases (Louw *et al.*, 2002). The compounds previously isolated from the bulb of *E. autumnalis* are eucomanalin, autumnariol and eucosterol (Van Wyk *et al*., 1997; Van Wyk & Gericke, 2000). The bulb was used for this study.

Distribution of *E. autumnalis*

E. autumnalis is widely distributed along the eastern parts of South Africa (Van Wyk *et al.*, 1997). It is predominantly found in the following provinces the Free State, KwaZulu-Natal, Eastern Cape, Mpumalanga, and Gauteng.

(a)

(b)

Figure 2.7 *Eucomis autumnalis* (a) flowers and leaves and (b) bulb (Courtesy: Van Wyk *et al*., 1997)

2.2. 8 *Gomphocarpus fruticosus* (L.) Aiton

G. fruticosus, formerly known as *Asclepias fruticosa* belongs to the family *Asclepiadaceae*. The plant is a small shrub of about 2 meters in height with slender stems and leaves attached opposite to each other. The yellowish-green flowers are borne in swinging clusters. Large round–shaped seed pods are attached to the stem (Figure 2.8). The leaves are used medicinally to treat headaches, stomach pain, tuberculosis and as an emetic. In the previous study quercetin glycosides, rutin and kaempferol and isorhamenetin were isolated from *G. fruticosus* (Heneidak *et a*l., 2006). Other compounds gomphoside, gomphacil, afroside, 5,6-didehydroafroside have been isolated from *G. fruticosus*. Gomphacil and afroside have been isolated from the leaves. Gomphoside is a cardiotonic agent (van Wyk *et al.,* 1997; van Wyk & Gericke, 2000). The leaves were used for the present study.

Distribution of *G. fruticosus*

It is widely distributed in all parts of South Africa.

Figure 2.8 *Gomphocarpus fruticosus* (Courtesy: Van Wyk *et al.* 1997)

2.2.1.9 *Heteromorpha arborescens* (Spreng) Cham. &. Schltdl

H. arborescens belongs to the family *Apiaceae*. The plant is commonly known as parsley tree. In Zulu and Xhosa it is known as "umbangandlala" and "mkatlala" in Sotho. It is a woody shrub or small tree approximately 15 metres in height. The bark is scale–like and is easily peels off. The flowers are yellow and the flower stalks arise from the same point. The bark and leaves (Figure 2.9) are medicinally used to treat headaches, fever, asthma, coughs, dysentery, infertility, abdominal pains and colic (Van Wyk *et al*., 1997; Lundgaard *et al*., 2008). The compounds falcarindiol, germacrene D, asacirin, sabinene and α-pinene have been isolated from *H. arborescens*. Falcarindiol has been isolated from the roots. Germacrene D, asacirin, sabinene and α-pinene have been isolated from the leaves. Falcarindol has been reported to have antifungal, antibacterial and analgesic activity (van Wyk *et al*., 1997; van Wyk & Gericke, 2000). Falcarindol has been reported to inhibit cyclooxygenase-1 (COX-1) (Lundgaard *et al*., 2008). *H. arborescens* has been reported to have antibacterial activity against Gram positive bacteria such as *Staphylococcus aureus*, *S. epidermidis*, *Bacillus subtilis*, *Micrococcus kristinae* and *Streptococcus faecalis* (Nkomo and Kambizi, 2009). The stem and the leaves were used for this study.

Distribution of *H. arborescens*

H. arborescens is extensively distributed in South Africa.

(a) (b)

Figure 2.9 *Heteromorpha arborescens* (a) bark (b) leaves and flowers (Courtesy: Van Wyk *et al.*, 1997)

2.2.10 *Plectranthus ecklonii* Benth.

Plectranthus ecklonii belongs to the family *Lamiaceae*. *P. ecklonii* is a shrub (Figure 2.10) which grows to about 2 metres tall growing best in a semi shade. *Plectranthus* species including *P. ecklonii* are traditionally used to treat stomachaches, nausea, vomiting and meningitis (Lukhoba *et al*., 2006). The leaves of *P. ecklonii* are used to treat skin infections. *P ecklonii* has been reported to be traditionally used for its anti-inflammatory and antifungal properties (Figueiredo *et al*., 2010). The aqueous extract of *P. ecklonii* has been reported to have antibacterial activity against *Streptococcus mutans* and *Streptococcus sobrinus*. In addition *P. ecklonii* also inhibited the formation of the biofilm of the two bacteria. The *P. ecklonii* extract further inhibited the glucosyltransferase and hence the extract may be used in the prevention of dental carie. The main compound present in *P. ecklonii* said to be responsible for this action is rosmarinic acid (Figueiredo *et al*., 2010). Other species of *Plectranthus* such as *P. laxiflorus* and *P. madagascariensis* have been reported to be used in South Africa for the treatment of tuberculosis

related diseases (McGaw *et al*., 2008). Ecklonoquinone A and B have been previously isolated from *P. ecklonii* (DNP, 2010). Other compounds that have been previously isolated from *P. ecklonii* are: 11-hydroxy-19-(methyl-buten-2-oyloxy)-abienta-5,7,9(11),13-tetraene-12-one),11-hydroxy-2α-(3,4dihydroxy-benzoyloxy)abieta-5,7,9(11),13-tetraene-12-one) and 7α-formyloxy-6β,12-dihydroxy-abieta-8,12-diene-11,14-dione (Van Zyl *et al*., 2007). These compounds were isolated from the leaves and were reported to have antiplasmodial activity (Van Zyl *et al*., 2007). The constituents of *Plectranthus* plants mainly diterpenoids have been found to have antimycobaterial activity (Rijo *et al*., 2010). Other compounds that have been previously isolated from *P. ecklonii* are cirsimaritin, ladanein and salvigenin (Grayer *et al*., 2010). Parviflorn D and F have been isolated from *P. ecklonii* and have been found to have antibacterial activity against *Mycobacterium tuberculosis* and *L. monocytogenes* (Nyila *et al*., 2009). The leaves were used for this study.

Distiribution of *P. ecklonii*

P. ecklonii is widely distributed in South Africa, Australia, New Zealand, Mexico and the United States.

Figure 2.10 Shrubs of *P. ecklonii* with leaves and purple flowers.

2.2.11 *Senecio inornatus* DC

S. inonartus belongs to the family *Asteraceae*. The plant is an herb with a upright stem. The leaves and stem are medicinally used to treat various illnesses such as chest pains, headaches, swellings, burns and sores. *S. inonartus* has been reported as one of the plants that is used in South Africa for the treatment of respiratory or chest complaints and coughing (McGaw *et al*., 2008). *Senecio* species are characterised by the presence of macrocylclic pyrrolizidine alkaloids such as toxic senecionine and the non-toxic plattyphylline. Senecionine has been isolated from the aerial parts of the plant. Senecionine has been reported to have antineoplastic activity (van Wyk *et al.*, 1997). The leaves were used for this study.

Distribution of *S. inornartus*

The plant is widely distributed in South Africa especially in the eastern part of the country (Brand *et al*., 2009).

2.2.12 *Tulbaghia violacea* Harv.

T. violacea belongs to the family *Alliaceae.* The plant is commonly known as wild garlic. In Zulu is known as "ishaqa". *T. violacea* is a bulbous plant with long leaves arising from the fleshy base. The purple flowers occur in groups at the tip of a thin stalk (Figure 2.11). The plant is characterised by a strong smell of garlic when damaged (Van Wyk *et al.*, 1997). The leaves and bulbs are medicinally used to treat fever, colds, asthma, tuberculosis (Van Wyk,

2008). Alliin is the main sulphur-containing compound of the whole plant. The bulb of *T. violacea* has been previously (Buwa & Afolayan, 2009) found to have antibacterial activity against *Escherichia coli*, *Bacillus cereus* and *Staphylococcus aureus*. The compounds, Bis[(methylthio)methyl] disulfide, Bis[(methylthio)methyl] disulfide 2,2-Dioxide, Bis[(methylthio)methyl] disulfide 4-Oxide and marasmine have been previously isolated from *T. violacea* (DNP, 2010). The leaves were used for this study.

Distribution of *T. violacea*

T. violacea is found mainly in the Eastern Cape and the southern parts of KwaZulu-Natal province.

Figure 2.11 *Tulbaghia violacea* with purple flowers (Courtesy: Van Wyk *et al*. 1997)

2.2.13 *Ziziphus mucronata* Wild.

Ziziphus mucronata belongs to the family *Rhannaceae.* It is commonly known as buffalo-

thorn. In Zulu it is known as "umhlakaniso", "umphafa" in Xhosa, "mokgalo" in Tswana and Pedi. It has a greyish-brown bark and sharp thorns are found on the twigs. The fruits are small round berries (Figure 2.12). The bark or leaves are medicinally used to treat coughs, chest problems, and diarrhoea, sore and glandular swellings. Decoctions of roots and leaves are applied externally to boils, sores and glandular swellings (Van Wyk *et al.*, 1997; Van Wyk and Gericke, 2000). In previous study *Z. mucronata* was found to possess antibacterial activity against *Enterococcus faecalis* and *Staphylococcus aureus* (MacGaw *et al.*, 2007). Also in another study methanol extracts of *Z. mucronata* have been found to have antibacterial activity against *B. subtilis, S. aureus and S. epidermis* (Rabe and Van Staden, 1997). Mucronine D and sanjoinine compounds have been isolated from the stem bark of *Z. mucronata* (Van Wyk *et al.*, 19 97; VanWyk and Gericke, 2000). The leaves were used for the present study.

Distribution of *Z. mucronata*

Z. mucronata is widely distributed in South Africa with the exception of the Western Cape province (van Wyk *et al*., 1997).

(a) (b)

Figure 2.12 *Ziziphus mucronata* (a) tree and (b) fruits (Courtesy: Van Wyk *et al.*, 1997)

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Chapter 3

Susceptibility testing of *Listeria monocytogenes* to extracts of selected South African medicinal plants

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Abstract

In South Africa, the antimicrobial activity of various indigenous plants has been studied extensively. Most of the work however, has focused on their activity against planktonic bacteria with less attention given to biofilms. Many organisms, including the opportunistic pathogen *Listeria monocytogenes* which is associated with severe infections in humans, occurs more frequently as biofilms. The aim of this study was to identify and select the plants that exhibit the best antilisterial activity, isolate the bioactive compounds and determine their effect on the architecture of listerial biofilms. Ethyl acetate or chloroform extracts of thirteen plants were investigated for antilisterial activity. The ethyl acetate extract of *Acacia karroo* and *Plectranthus ecklonii* showed the best antilisterial activity (among the plants tested) exhibiting a minimum inhibitory concentration (MIC) activity of 3.1 mg/ml and 0.5 mg/ml respectively and were therefore further selected for the identification of bioactive compounds. Column chromatographic purification of the ethyl acetate extracts of the leaves of *A. karroo* led to the isolation of three known pure compounds namely β-sitosterol, epigallocatechin and epicatechin. The confocal scanning laser microscopy (CSLM) showed that the biomass of the listerial biofilm was reduced when the isolated compounds were added. The aggregation of cells which were exposed to β-sitosterol and epigallocatechin was reduced from 25µm as observed in untreated cells to < 10 µm in diameter.

3.1 Introduction

A number of medicinal plants have been reported by previous researchers to have antilisterial activity (Shan *et al*., 2007; Alzoreky, 2009; Koochak, *et al*., 2010). However, South African plants have been reported to have activity against other microorganisms such as *Mycobacterium tuberculosis*, *Staphylococcus aureus*, *Bacillus subtilis*, *B. cereus, E.coli, Klebsiella pneumoniae,* etc. (Rabe and Van Staden, 1997; Lall and Meyer, 1999, Lall and Meyer, 2000; Mangena and Muyima, 2009; Buwa and Afolayan, 2009). Not much work has been done by scientists towards exploring the potential of South African plants for antilisterial activity.

Local plant species such as *Artemisia afra, Acacia karroo, Ziziphus mucronata*, *Eucomis autumnalis,* have been used extensively for the treatment of listeriosis related symptoms (Van Wyk *et al*., 1997; Van Wyk and Gericke, 2000) while globally, *Camellia sinensis* (Si *et al*., 2006), *Ruta graveolens* (Alzoreky and Nakahara, 2003), *Mutisia acuminata var. acuminata* (Catalano *et al*., 1998) have also been found to have antilisterial activity.

Current research on these medicinal plants has placed greater emphasis on their antimicrobial activity against free floating cells (planktonic) with less focus on biofilms which are associated with severe infections (Quave *et al*., 2008). Bacterial biofilms are more resistant to the action of antimicrobial and disinfectant agents (García-Almendárez *et al*., 2007). The resistance (Nichterlein *et al*., 1998; White *et al*., 2002) has resulted in the need for multidrug treatment associated with high side effects and increased toxicity (Cone *et al*., 2003; Gleckman & Borrego, 1997). The aim of the study was to identify and select the plants that exhibited the best antilisterial activity, isolate the bioactive compounds and determine their effect on the architecture of listerial biofilms.

3.2 Materials and Methods

3.2.1 Plant material

Thirteen South African medicinal plants were collected from Gauteng and the Free State. Different parts of the plants (i.e. leaves, stem, bark and, roots) were collected (as shown in Table 3.1). The plant collection was based on information received from experienced traditional healers, elderly indigenous people (who are experts on traditional medicine) and from literature. Plants were taken to HGWJ Schweickerdt herbarium at the University of Pretoria for identification. Herbarium specimens were preserved in the above herbarium.

3.2.2 Preparation of crude plant extracts

One fairly polar solvent ethyl acetate and another solvent of medium polarity (chloroform) were selected. Due to finance constraints water and ethanol solvents were not included (This would had increased the number of samples to be analysed). Ethyl acetate and chloroform extracts of each plant sample were prepared. To obtain these extracts one hundred grams (100 g) of fresh plant material was homogenised and extracted with ethyl acetate or chloroform. The extract was filtered and concentrated. The residue was later dissolved in 10% DMSO to a final stock concentration of 50 mg/ml.

3.2.3 Bacterial strain and inoculum preparation

The pathogenic strain of *Listeria monocytogenes* (LMG 21263) used in this study was obtained from the Department of Pharmaceutical Sciences at Tshwane University of Technology. It was activated by transferring a loopful from the Brain Heart Infusion (Merck) slants into

Tryptone Soya Broth (Merck), followed by incubation at 37°C for 24 hours. The bacterial counts of the standardized culture were confirmed by plating out on TSA (Merck) plates and incubating at 37°C for 24 hours. Stock cultures were maintained at -70 °C (Alzoreky & Nakahara, 2003).

3.2.4 Antimicrobial bioassay

Disc diffusion method

The disc diffusion method as described by Alzoreky & Nakahara (2003) was used for testing the susceptibility of *L. monocytogenes* to the plant extracts. Two hundred microlitres of prepared culture (10^6 CFU/ml) was spread on surfaces of Mueller–Hinton agar. Sterile filter paper discs (10 mm in diameter) were impregnated with 50 microlitre (50 mg/ml) of the extracts. Erythromycin (150 µg/ml) was used as a positive drug control. The DMSO (2.5%) was used as control to test the inhibition of the bacteria. Spread plates were then kept at ambient temperature for 30 minutes to allow diffusion of the extracts prior to incubation at 37 °C for 24 hours.

Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

Extracts (ethyl acetate or chloroform extracts of *Acacia karroo*, *Eucomis autumnalis*, *Drimia altissima*, *Aloe arborescens*, *Plectranthus ecklonii* and *Senecio inonartus*) which showed antilisterial activity in the initial screening using the disc diffusion method were further evaluated to determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) using 96-well microtitre plates. The micro dilution method as described by Eloff (1998) was used. Briefly, the extracts were first dissolved in 10 % DMSO and then added to Tryptone Soya Broth (TSB) to obtain a final concentration of 25 mg/ml in the first well. The DMSO (2.5%) was used as control to test the inhibition of the bacteria. A serial double dilution

was performed to obtain a concentration range of 25- 0.01 mg/ml for the extracts. For compounds the antibiotic erythromycin (Merck) at concentrations ranging from 150 - 0.29 µg/ml served as a positive drug control. Hundred microlitres of bacterial inoculum 10^6 CFU/ml of *L. monocytogenes* was added to the wells thereafter, the plates were then incubated at 37 °C for 24 hours. After 24 hours incubation microbial growth was tested by adding 40 µl of (0.2 mg/ml) *p*-iodonitrotetrazoilium violet (INT) (Sigma-Aldrich, South Africa) to the micro titre wells and re-incubated at 37 °C for 1 hour. Change in colour to orange red indicated that the cells were still viable. The MIC was defined as the lowest concentration of the extract that caused no colour change. To determine the minimum bactericidal concentration (MBC) against *L. monocytogenes*, fifty microlitres of the sample (from the wells which did not show bacterial growth during MIC determination) were transferred into 150 microlitres of fresh TSB on the new plates. The plates were then re-incubated for another 24 hours and the MBC (lowest dilution of extracts with no growth after 24 hours incubation at 37 °C) was determined according to Reimer *et al*. (1981).

A. karroo and P. ecklonii warranted further tests to be done for the isolation and identification of the active compounds. The description on the isolation of compounds from *P. ecklonii* is dealt with in Chapter 4. The crude extract of *A. karroo* and *P. ecklonii*, not only had the best MICs value but also exhibited the lowest MBC values (Table 3.2).

Identification of active compounds

The leaves of *A. karroo* (1.2 kg) were extracted with ethyl acetate (2 x 2 L) for 48 hours at room temperature (± 25 °C). The ethyl acetate extract was filtered and concentrated in a vacuum. The total extract (72.7 g) was subjected to silica gel column chromatography (CC) using hexane/ethyl acetate mixtures of increasing polarity (0%, 20% (v/v), 40%, 70 %, 100 % ethyl acetate) and

at the end 100% methanol (MeOH) was added (Figure 3.1). Similar fractions were combined according to the TLC profile into 12 main fractions (Figure 3.2).

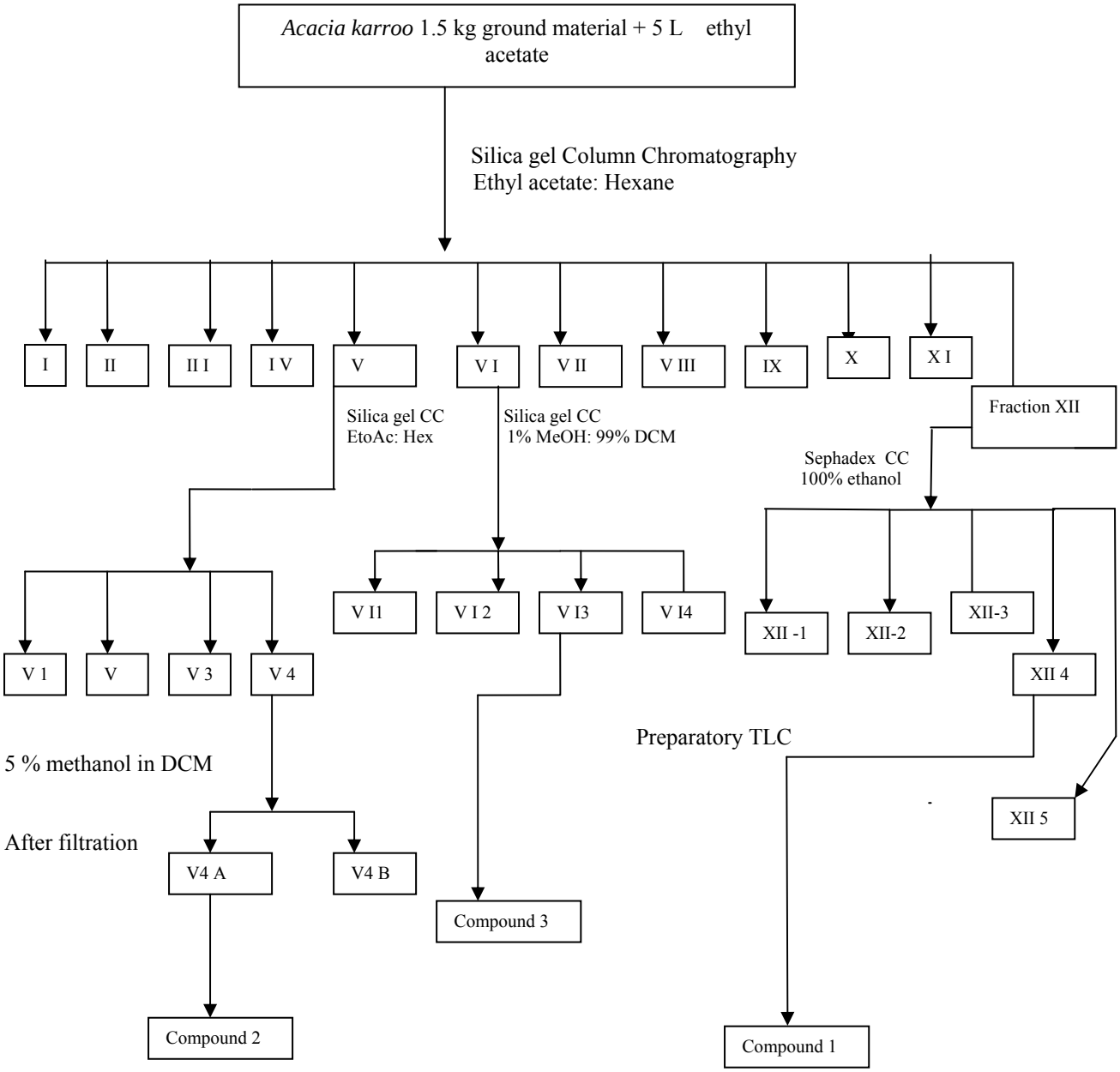
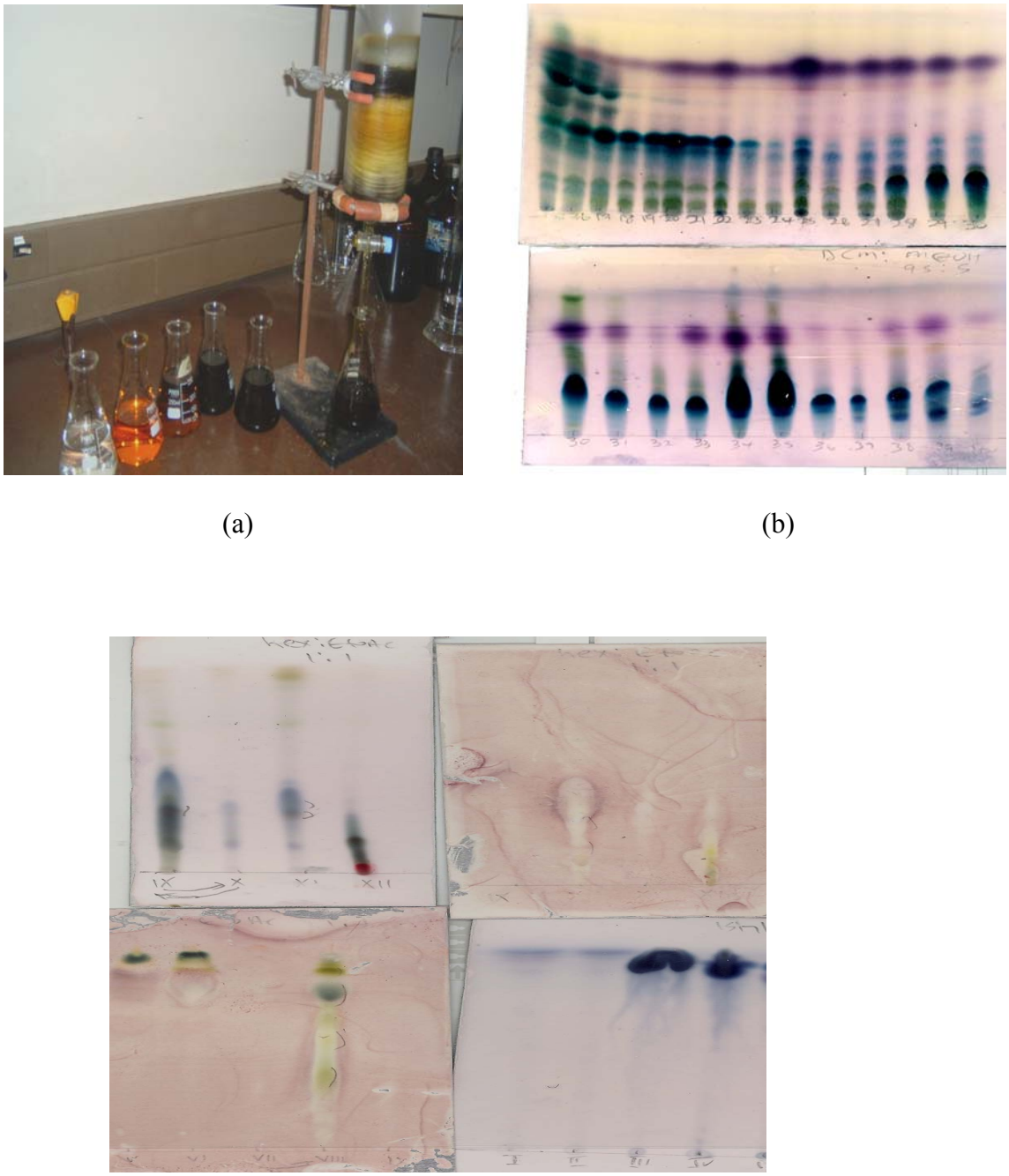


Figure 3.1 Schematic representation of the isolation process of the bioactive compounds from *Acacia karroo*

Direct bioassay on the TLC plate was done by applying 20 µl of the plant extract (50mg/ml) to the silica gel 60 F_{254} plate (Simonovska & Vovk, 2000). The plates were developed using ethyl acetate: ethanol (9:1, v/v) eluent and then dried carefully. The 24-hour *L. monocytogenes* in TSB was centrifuged at 1000 rpm for 15 min. The supernatant was discarded and the pellet dissolved in fresh nutrient broth. A fine spray was then used to apply the bacterial suspension onto the TLC plates according to Meyer & Dilika (1996). The plates were then incubated at 37 °C for 24 hours. After incubating, the plates were sprayed with 0.2 mg/ml INT. (The viable bacteria would change the INT salt on the TLC plate into deep coloured formazan) (Hamburger & Cordell, 1987). Fractions IV, V and XII showed good zones of bacterial inhibition in a direct bioautographic assay and hence were subjected to further column chromatographic purification. Fraction IV was subjected to a Sephadex LH-20 column using 100 % ethanol to give compound **1** (yield 24 mg, 0.002 %). Fraction V was subjected to silica gel chromatography using hexane: ethyl acetate (9:1) (v/v) as eluent which resulted in the pure compound **2** (yield 600 mg, 0.05 %). Fraction XII, under the same conditions gave pure compound **3** (yield 128.4 mg, 0.017 %). ^{1}H NMR and ^{13}C NMR spectra were recorded using a Brucker ARX 300 or a Brucker Avance DRX 500 MHZ using $CDCl_3$, and DMSO-d_6 as solvents which led to the identification of the purified compounds.



(a) (b)

(c)

Figure 3.2 (a) Silica gel column chromatography of the ethyl acetate extract of *A. karroo* (b) and (c) direct bioassay on the TLC plates.

Cytotoxicity test

The cytotoxicity of crude extracts and pure compounds isolated from *A. karrroo* was conducted by Ms Karlien le Roux following the method as described by Mathabe *et al*. (2008). Cytotoxicity was investigated by using XTT-based colorimetric assay Cell Proliferation Kit II (Roche Diagnostics GMbH). The final concentration for crude extract in the wells ranged from 3.125 to 400 µg/ml. for pure compounds ranged from 1.5 200 µg/ml . The positive drug 'Zearalenone', at a final concentration of 1.25 µg/ml, was included as a positive control. The final percentage of DMSO was 2% (for crude extract) and 0.5% (for pure compound). The concentration of the pure compound at which 50% (IC_{50}) of the Vero cells were alive until the 4th day was considered to be the highest concentration which is non-toxic to the cells. After incubation the absorbance of the colour was spectrophotmetrically quantified using ELISA plate reader, which measured the optical density at 490 nm with a reference wavelength of 690 nm. Assay was carried out in triplicate. The IC_{50} was defined as the concentration of the compounds at which absorbance was reduced by 50%. The results were statistically analysed with the 'GraphPad Prism 4' statistical programme. The analysis was done by selecting the sigmoidal dose response (variable slope) curve fit as well as the 95% confidence interval option. The analysis was limited to values between 0 and 100. The IC_{50} value was then calculated by the program.

Confocal Scanning Laser Microscopy (CSLM) of the crude extracts of A. karroo and its compounds against listerial biofilms

A modified Kives *et al.* (2005) method was used for investigating the effect of the antilisterial extracts of *A. karroo* and its purified compounds on listerial biofilm formation. Briefly, a standardized overnight culture was allowed to develop a biofilm on glass slides that had been previously coated with 100 µl TSB to provide nutrients for the adhering bacteria (Chae & Schraft, 2000). These were then placed in sterile petri dishes and incubated at 37 °C for a further 24 hours. After incubation these glass slides were either left untreated (negative control), treated with erythromycin (positive control) or the ethyl acetate extract of *A. karroo* (1.0 mg/ml) or the pure compounds (500 µg/ml). After 24 hours incubation, the slides were removed from the incubator and the samples prepared for viewing with the Zeiss LSM 510 META (Carl Zeiss, Jena, Germany). The sample was prepared as follows: 1µl of propidium iodide was combined with 19 µl of sterile double distilled water and poured over the biofilm containing slide. The use of the dye, propidium iodide, facilitated the viewing of the listerial biofilm under the confocal scanning laser microscope. The presence or reduction of the biofilm on the glass slide was the indication of whether the samples were active or not against the *L. monocytognes* cells. This will be illustrated by the images from the CSLM.

3.3 Results and Discussion

Three known compounds were isolated from ethyl acetate extract of *A. karroo*. The respective NMR spectra as follows:

Epicatechin (Compound **1)**

This compound gave a reddish brown spot using vanillin /H_2SO_4 reagent on TLC was

eluted from Fraction XII. The compound was identified on the basis of ^{1}H-NMR data (Figure 3.3). The ^{1}H-NMR data of **1** exhibited signals identical with that of epicatechin which exhibited signals of 6 protons, at δ 4.85 assigned to H-2, a proton signal δ 4.18 ppm, to H-3, and the two protons signals at 2.74 and 2.80 ppm were assigned to protons 4α and 4β respectively, signal at δ 6.01 was assigned to H-6 and the signal at 5.90 to H-8 (Okushio *et al*., 1998). Aromatic signals at δ_H 7.02, 6.75, and δ_H 6.80 corresponds with that of a B-ring. The basic structure was derived as a 3,3', 4',5,7-pentahydroxyflavan and the broad proton singlet at δ_H 4.82 suggested a epicatechin (Sun *et al*., 2006). Compound **1** belongs to the flavonoid group of compounds. Compound **1** has been previously isolated from the bark of *Pterocarpus marsupium*, (Chakravarthy and Gode, 1985; DNP, 2010), also Chinese and Korean green tea, (Row and Jin, 2006; Si *et al*., 2006), *Litchi chinensis* and cocoa (Sun *et al*., 2006; DNP, 2010), berries of *Vitis vinera* (Anastasiadi *et al*., 2009). The compound is regarded an anti-inflammatory and anti-ulcer agent. It has been reported to have hepatotropic and antioxidant activities (DNP, 2010). The compound had been previously tested on *L. monocytogenes* and did not show activity (Anastasiadi *et al*., 2009).

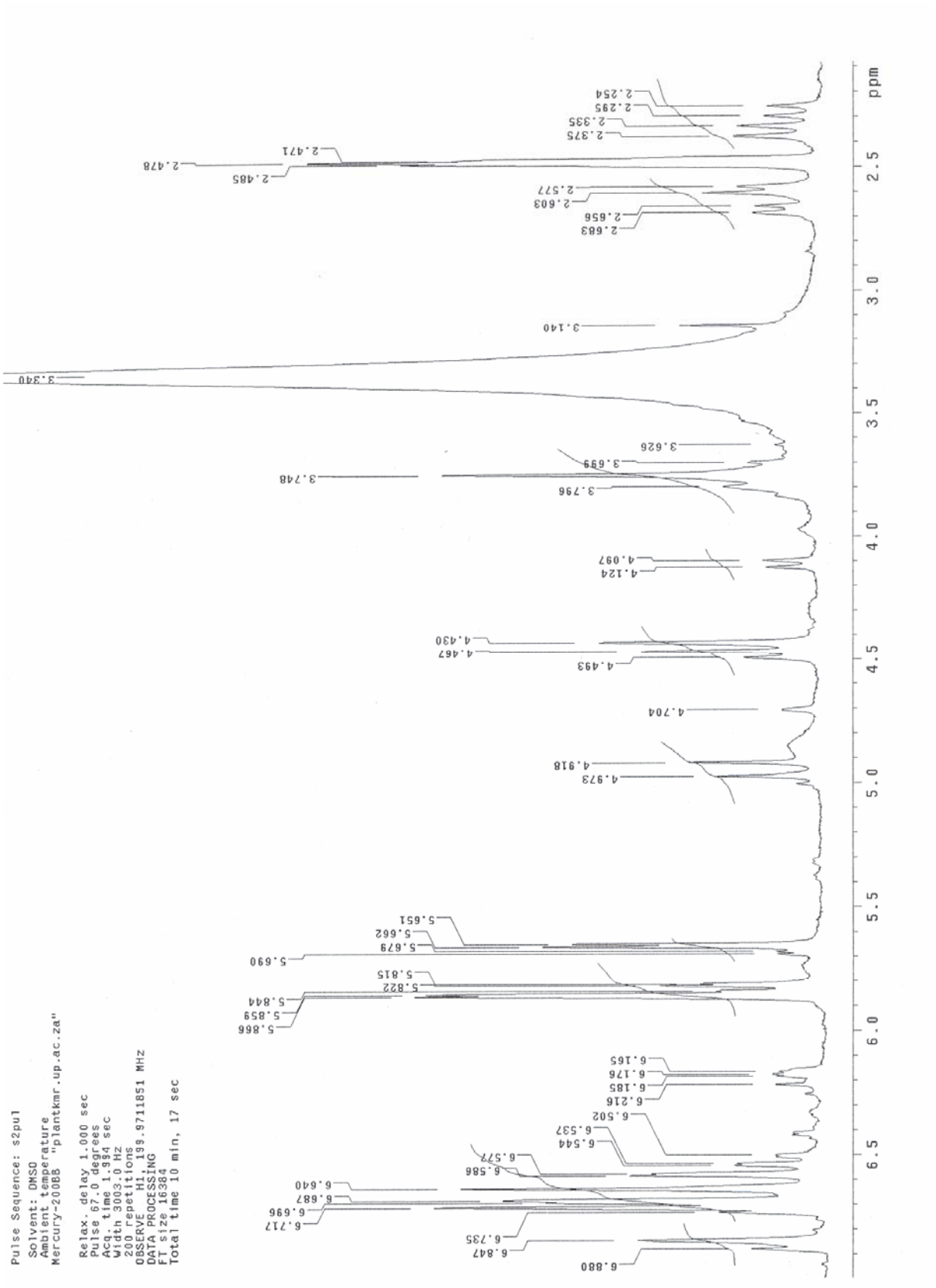


Figure 3.3 [1]HNMR spectrum of compound **1** (epicatechin) isolated from *A. karroo*.

β –sitosterol (Compound **2**)

From Fraction V one pure compound (**2**) was obtained. The compound was isolated as colourless needle. The compound was identified on the basis of ^{1}H-NMR data (Figure 3.4). ^{1}HNMR spectra showed peaks at δ_H 5.35 corresponding to H-6, δ_H 3.79 to h-3 in addition to six methyl group signals at δ_H 1.00, 067 (s each, Me-19,-18), 0.90, 0.85, 0.80 (d each Me-21, 26, 27) and 0.87 (t, Me-29). The forementioned data with the ^{13}C NMR (Figure 3.5) indicated the presence of β–sitosterol, the common triterpene compound. This was supported by the comparison with those reported in literature (Moghaddam *et al*., 2007). Compound **2** has previously been isolated from *Raulinoa echinata* Cowan (Biavatti *et al*., 2001; Chattopadhyay *et al*., 2002; Misra *et al*., 2008) and other *Acacia* species such as *A. farnesiana* (Garcia *et al*., 2006). Compound **2** has also been previously isolated from the aerial parts of *Satureja khuzistanica*, *Mentha cordifolio* Opiz , *Vitex negundo* and *Croton membranaceus* (Villaseñor *et al*., 2002; Chandramu *et al*, 2003; Moghaddam *et al*., 2007). Compound **2** has been reported to have antibacterial activity against Gram positive bacteria such as *S. aureus*, *B. subtilis* as well as Gram negative bacteria, *E. coli* and *Pseudomonas aeruginosa* (Sanches *et al*., 2005; Bayor *et al*., 2009). To the knowledge of the author no report of compound tested against *L. monocytogenes*.

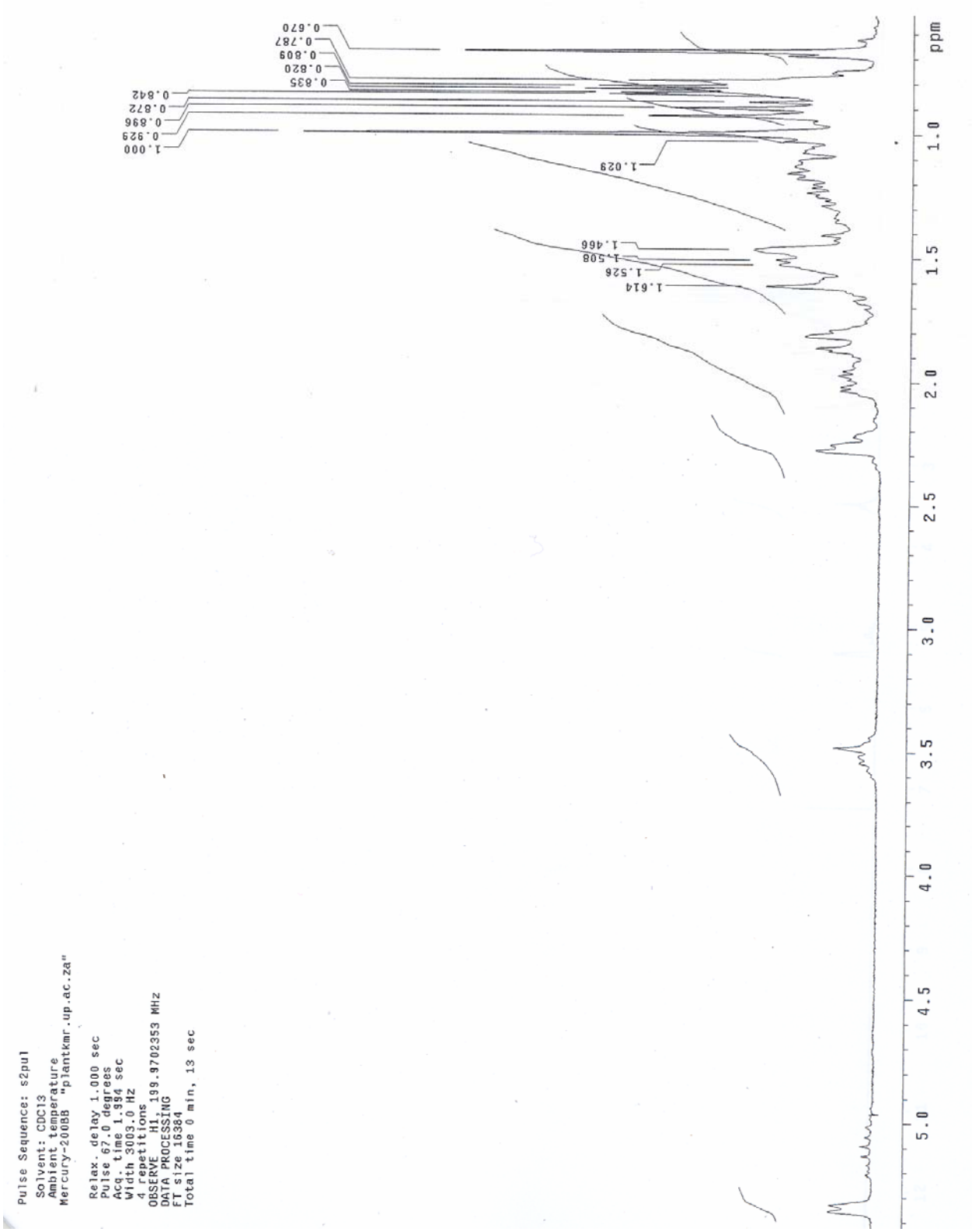


Figure 3.4 [1]HNMR spectrum of compound **2** (β-sitosterol) isolated from *A. karroo.*

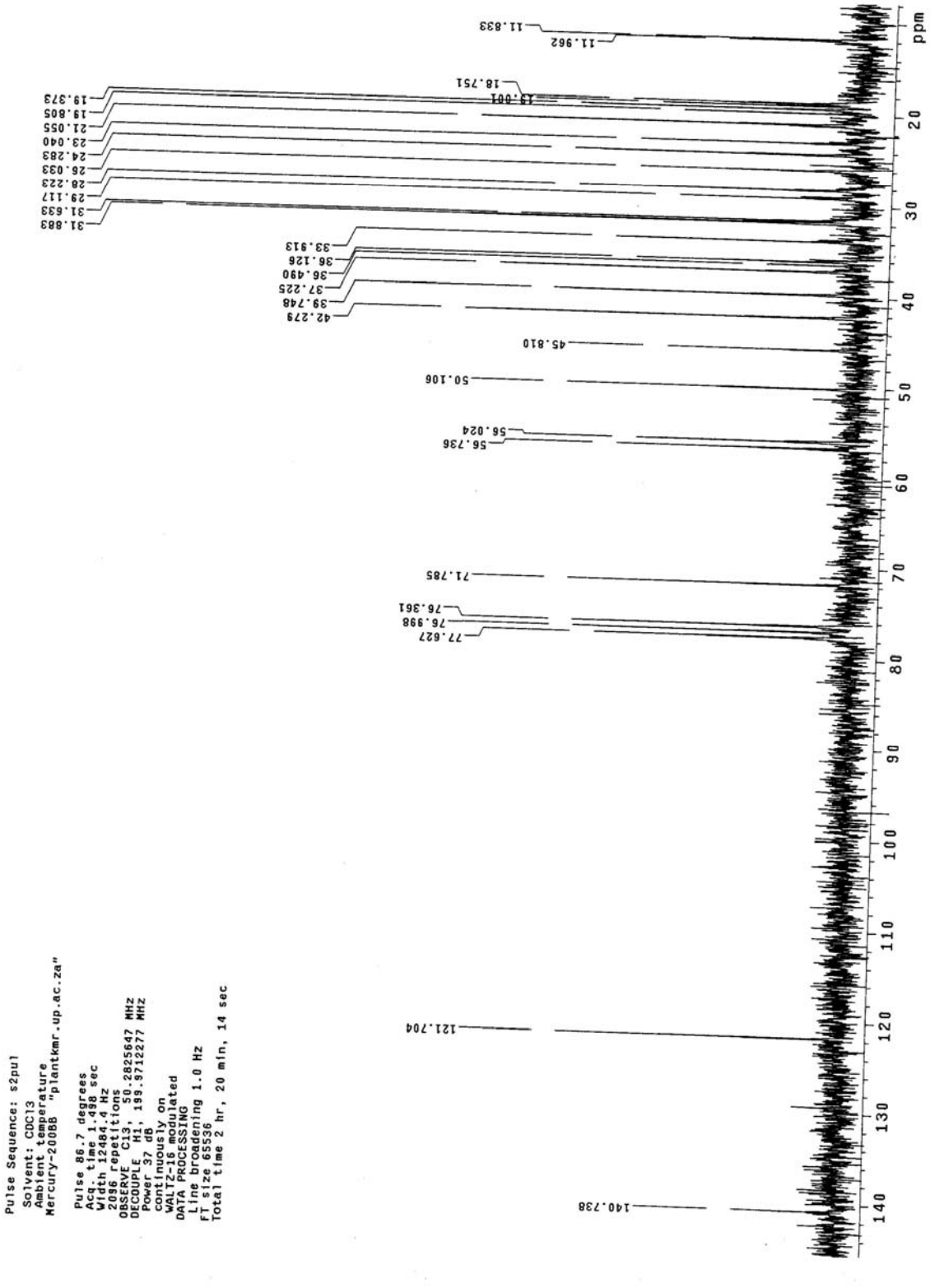


Figure 3.5 ^{13}CNMR spectrum of compound **2** (β-sitosterol) isolated from *A. karroo.*

Epigallocatechin (Compound **3**)

Fraction IV contained one pure compound **3** as determined by TLC and other spectroscopic methods. It was obtained by brown powder (24 mg). The ^{1}H-NMR spectrum (Figure 3.6) which showed four aromatic protons at δ_H 5.68 (d, J = 2.3), 5.87 (d, J = 2.3), 6.38 (2H, d, J= 0.6), two methyl protons at δ_H 4.86 (d, J= 0.8 and 3.77 (s br), methylene protons at δ_H 2.45 (J = 4.4) and 2.33 (J= 3.2). The ^{13}C-NMR spectra (Figure 3.7) indicated the presence of two methyl carbons attached to an oxygen function (δ_C 78.6, 65.6), a methylene carbon (δ_C 28.8 t), 12 aromatic carbons δ_C 156.4 (s), 156.2, 156.2, 155.3 (d), 145.7 (x 2C, d each), 132.5, 129.9., 106.0 (x 2C, s each), 99.0. The coupling constant between protons at δ_H 4.86 and 3.77 is 2.3=Hz which indicated β relative configuration. The above spectroscopic data indicated that compound **3** is epigallocatechin. Compound **3** is a flavonoid. Compound **3** has been previously isolated from green tea (Si *et al*., 2006; Gamberucci *et al*., 2006) and from other plants (Sivakumaran *et al*., 2004; Chirinos *et al*., 2008). Compound **3** has also been previously isolated from the bark of *Platanus orientalis* (DNP, 2010). Compound **3** has also been previously isolated from *Elaegnus glabra* Thumb and *Vinitis vinera* (Mori *et al*., 1987; Hamilton-Miller, 1995; Souquet *et al*. 2000). Compound 3 has been reported to have antibacterial activity against *S. aureus*, *S. epidermidis* and Gram negative bacteria, *Proteus vulgaris* (Mori *et al*. 1987; Nishino *et al*, 1987; Taguri *et al,* 2004). Compound **3** has also been reported to have antimicrobial activity against 10 strains of *Pseudomonas* (Fukai *et al*., 1991). According to literature epigallocatechin has not been tested on *L. monocytogenes*. The chemical structure of compounds **1**, **2** and **3** are given in Figure 3.8.

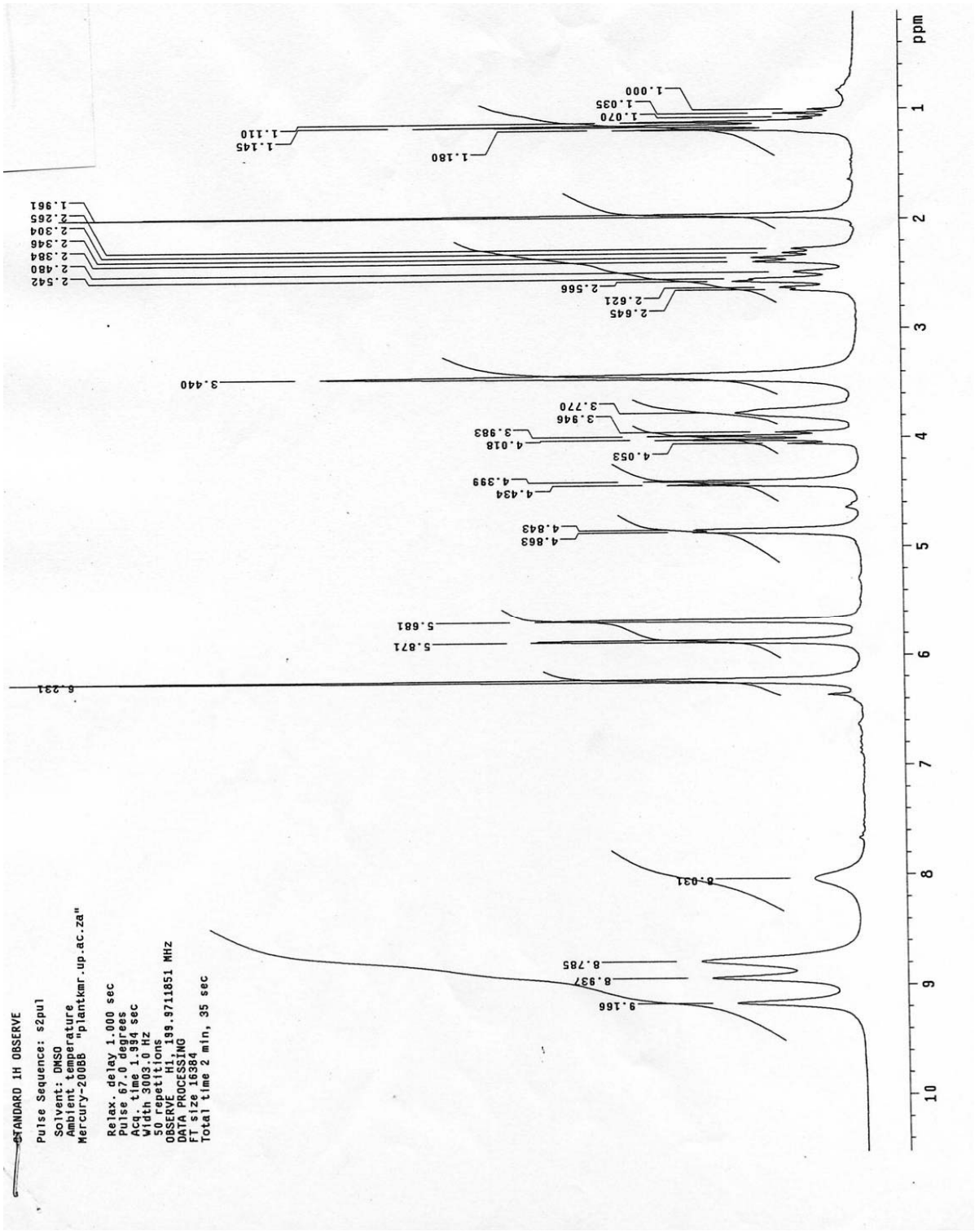


Figure 3.6 ^{1}HNMR spectrum of compound **3** (epigallocatechin) isolated from *A. karroo.*

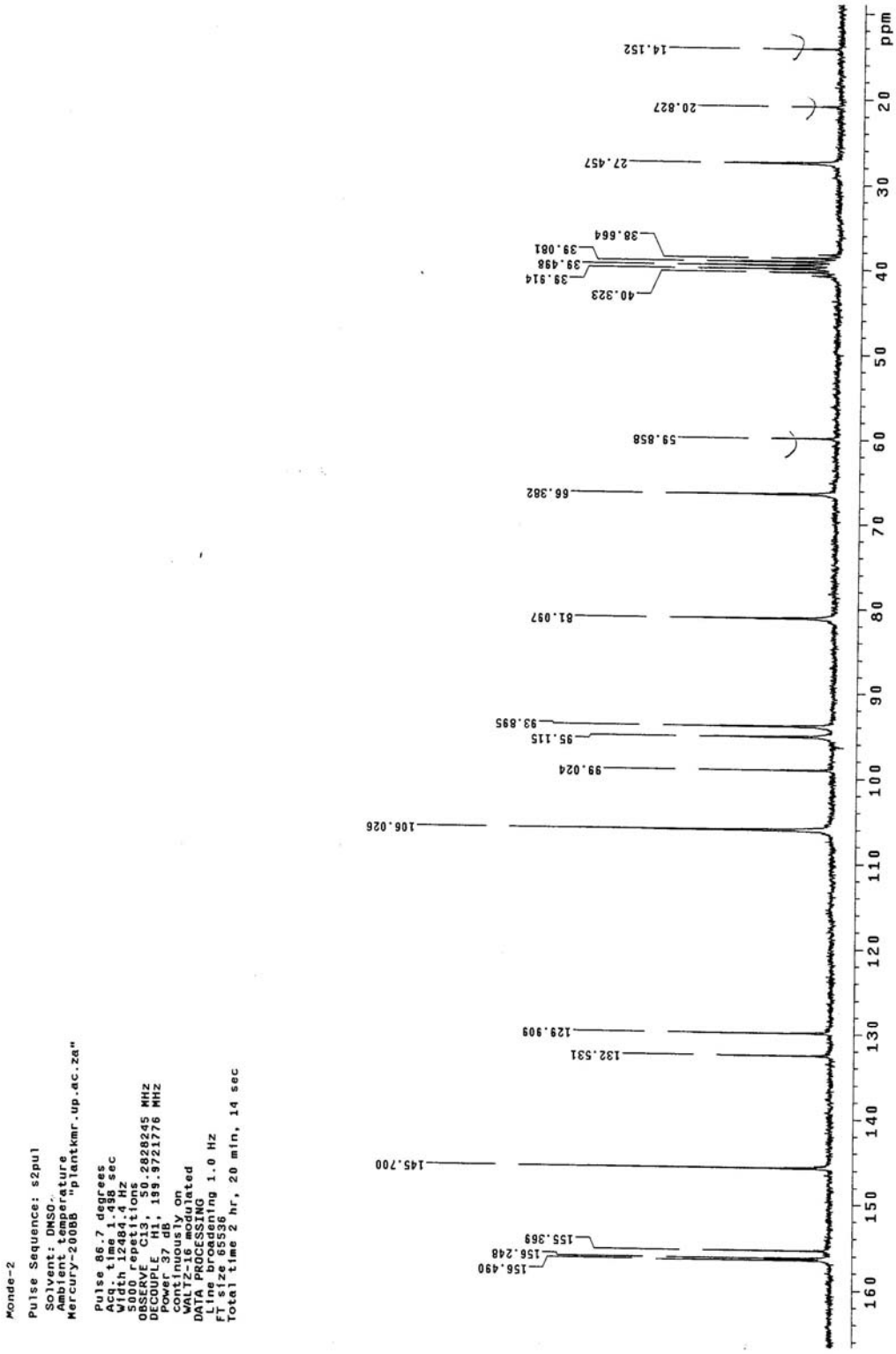
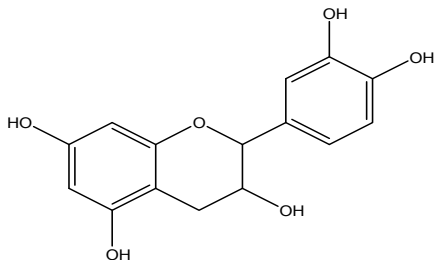
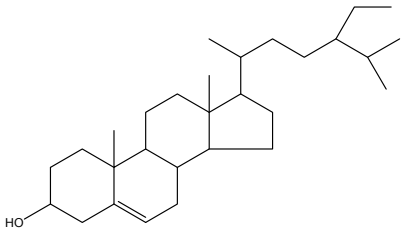


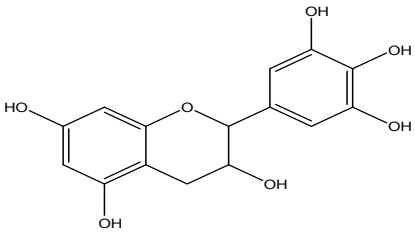
Figure 3.7 ^{13}CNMR spectrum of compound **3** (epigallocatechin) isolated from *A. karroo.*



1



2



3

Figure 3.8 Chemical structures of compounds isolated from the ethyl acetate extract of the leaves of *A. karroo*, epicatechin(**1**) , β-sitosterol (**2**), epigallocatechin (**3)**

Arslan and Özdemir (2008) used the disc diffusion method as an initial screening assay to determine the antilisterial activity of selected antibiotics. The researchers found that 12% of *Listeria* species isolated from cheese were resistant to commonly used antibiotics such as penicillin. In contrast, multidrug treatment with trimethoprim-sulfamethoxazole resulted in almost 98% sensitivity by the *Listeria* species used. In this study, of the 13 plant species tested only five showed activity against *L. monocytogenes* in the disc diffusion bioassay. Five plants namely *A. karroo* (ethyl acetate extract), *P. ecklonii* (ethyl acetate extract), *Senecio inonartus* (ethyl acetate extract), *Aloe arborescens* (ethyl acetate extract) and *E. autumnalis* (chloroform extract) exhibited good minimum bactericidal activity against *L. monocytogenes*, and the MBC ranging from 0.5 mg/ml to 12.5 mg/ml. The most active plant extracts against *L. monocytogenes* were *A. karroo* (14 mm inhibition) and *P. ecklonii* (15 mm inhibition) while *S. inonartus*

and *A. arborescens* also had limited inhibition at a concentration of 50 mg/ml (Table 3.1). The 50 mg/ml concentration of the plant extracts used in the disc diffusion assay in this study is low compared to other studies (Doughari *et al*., 2007; Kumar *et al*., 2007) where 100 mg/ml of plant extracts was used *in vitro* for antilisterial activity. The crude extracts that showed inhibitory activity in the disc diffusion bioassay were selected for further tests against *L. monocytogenes*. The MIC value for the *A. karroo* crude extract at 3.1 mg/ml showed the highest antilisterial activity (Table 3.2) and this is similar to results obtained by Alzoreky and Nakahara (2003) on Asian plant extracts. Alzoreky and Nakahara (2003) reported the MIC values ranging from 1320 – 2640 mg/L of buffered methanolic extract of *Artemisia absithium* and eighteen other plants against *L. monocytogenes*. In contrast, the alcohol extract of *Rhus coriaria* had an MIC of 2.5 mg/ml (Nasar-Abbas and Halkman, 2004). The ethyl acetate extracts of *P. ecklonii* and *A. karroo* showed the best antilisterial activity (among the plants tested) exhibiting a minimum inhibitory concentration (MIC) of 0.5 and 3.1 mg/ml respectively and were thus selected for the identification of their bioactive compounds.

Table 3.1 Antilisterial activity of the plant extracts (50 mg/ml) against *Listeria monocytogenes* (LMG 21263) as determined by the disc method.

Plant species	Plant part used [a]	Zone of inhibition (mm) Ethyl acetate	Zone of inhibition (mm) Chloroform	Voucher specimens
Aloe arborescens	L	9	8	MN 5
Acacia karroo	L	14	12	MN 15
Artemisia afra	L	NZ[b]	NZ	MN 7
Clivia miniata	W	NZ	NZ	MN 3
Datura stramonium	L	NZ	NZ	MN 8
Drimia altissima	R	NZ	NZ	MN 14
	L	NZ	NZ	
Eucomis autumnalis	B	12	13	MN 11
Gomphocarpus fruticosus	L	NZ	NZ	MN 1
Heteromorpha arborescens	S	NZ	NZ	MN 4
	L	NZ	NZ	
Plectranthus ecklonii	L	15	12	PRU 96396
Senecio inornatus	L	8	8	MN 9
	S	8	NZ	
Tulbaghia violacea	L	NZ	NZ	MN 12
Ziziphus mucronata	L	NZ	NZ	MN 10
Erythromycin (150 µg/ml) (drug control)			14 mm	

[a]plant part used: (W) whole plant; (L) leaves; (R) roots; (B) bulb; (S) stem

[b]NZ : No zone of inhibition observed (extract not active against *L .monocytogenes*)

Compound **1** a flavonoid did not exhibit good activity against *L. monocytogenes*. This is in line with the previous study where it was reported that Compound **1** did not inhibit the growth of *L. monocytogenes* (Anastasiadi *et al*., 2009). Compound **1** has been reported to have antibacterial activity against *S. aureus* and *E. coli* (Akiyama *et al*., 2001; Si *et al*., 2006). Compound **2** a terpene, and compound **3** a flavonoid exhibited good MIC against *L. monocytogenes* (Table 3.2). Compound **2** has been reported to have antibacterial activity against Gram positive bacteria such as *S. aureus*, *B. subtilis* as well as Gram negative bacteria, *E. coli* and *Pseudomonas aeruginosa* (Sanches *et al*., 2005; Bayor *et al*., 2009). Compound **3** has been previously reported to have antibacerial actvity against *Proteus vulgaris* and *S. Aureus* (Mori *et al*., 1987). The flavonoid, pinocembrin has been previously reported to have antilisterial activity (Välimaa *et al*., 2007). Other flavonoids that have been reported to exbibit both bactericidal and bacteriostatic activity against several strains of *L. monocytogenes* are hydroxycinnnamic acids (Puuppoene-Pimiä *et al.,* 2005). It has also been previously reported that cinamic, *p*-coumaric, ferulic, cafeic, carsonic acids as well as luteolin showed activity against *L. monocytogenes* (Del Campo *et al*., 2003; Wen *et al*., 2003). The relatively low MICs exhibited by these flavonoids warrants further investigation for use as natural antilisterial medicinal products. Epicatechin (**1**) exhibited the IC_{50} value of >200 µg/ml. β–sitosterol killed less than 50% cells at highest concentration the IC_{50} value was found to be > 200 µg/ml.

Although it had been previously reported that compounds with the lowest MICs are toxic to green monkey Vero cell line (Zentz *et al*., 2004), this study has shown that not only did the phytosterol, β –sitosterol (**2**) show the lowest MIC (Table 3.2), it also has a moderate, 50 % cytotoxic concentration (IC_{50}) value as compared to the control drug (Figures 3.9 and 3.10).

The selectivity index (SI = IC_{50}/MIC) was found to be to 6.45 and 0.466 for β –sitosterol (**2)** and epigallocatechin (**3**) respectively. β-sitosterol (**2**) had the highest SI value compared to

the other isolated compounds in the present study. β-sitosterol (**2**) was found to be more active and more selective than epigallocatechin. This is in accordance with the literature where the compound with the highest activity was also found to be more selective (high SI value) than the compound with less activity (Kovala-Demertzi *et al.*, 2009).

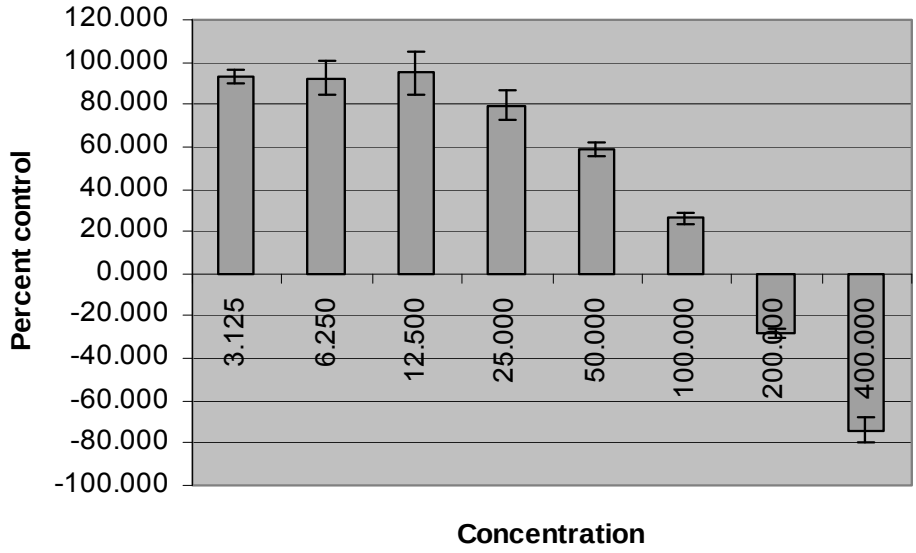


Figure 3.9 Dose response curve of *A. karroo* ethyl acetate extracts against Vero cell lines.

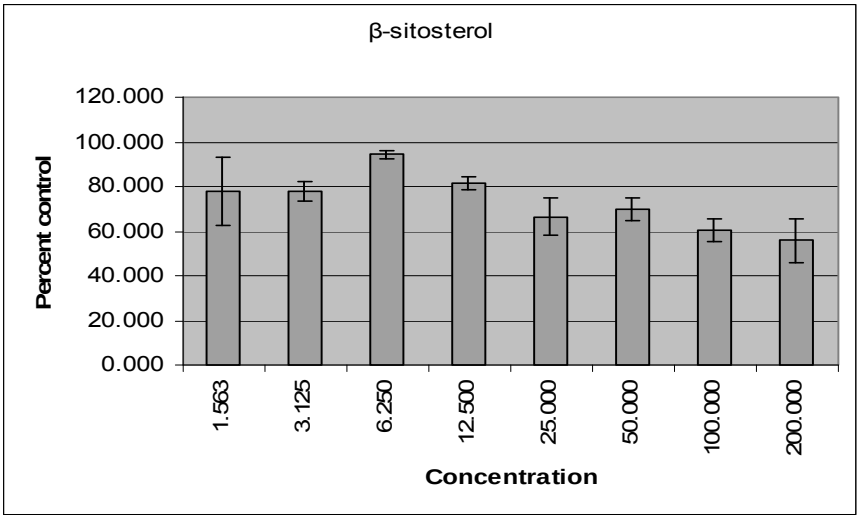
Table 3.2 The minimum inhibitory concentration and the minimum bactericidal concentration of the crude extracts of *A. karoo*, *E. autumnalis*, *S. inonartus*, *A. arborescens* and *P. ecklonii* against *L. monocytogenes* and their fifty percent inhibitory concentration against Vero monkey cell lines.

Compounds/extracts	MIC (mg/ml)	MBC (mg/ml	*IC_{50} (µg/ml) ± SD	SI value = IC_{50} / MIC
Epicatechin (**1**)	> 0.5	> 0.5	>200.0	NA
β-sitosterol (**2**)	0.031	0.125	> 200	6.45
Epigallocatechin (**3**)	0.062	0.25	28.91 ± 1.525	0.466
A . karroo (ethyl acetate) (chloroform)	3.1 6.25	3.1 6.25	45.49 ±7.86 NT [a]	0.015 NA
P. ecklonii (ethyl acetate) (chloroform)	0.5 6.25	1.0 6.25	30.125 NT	0.06 NA
E . autumnalis (ethyl acetate) chloroform	12.5 12.5	12.5 12.5	NT NT	NA NA
S. inornatus (ethyl acetate) (chloroform)	12.5 12.5	12.5 12.5	108.4 ± 0.995 99.94 ± 4.191	0.009 0.008
D. altissima (ethyl acetate) (chloroform)	12.5 12.5	12.5 12.5	NT NT	NA NA
A. arborescens (ethyl acetate) (chloroform)	12.5 12.5	12.5 12.5	>400.0	0.032 NA
Erythromycin (antibacterial drug control)	1.7×10^{-3}	1.7×10^{-3}	14.38	8.46
Zearalenone (positive drug for cytotoxicity)	NA	NA	2.318± 0.301	NA

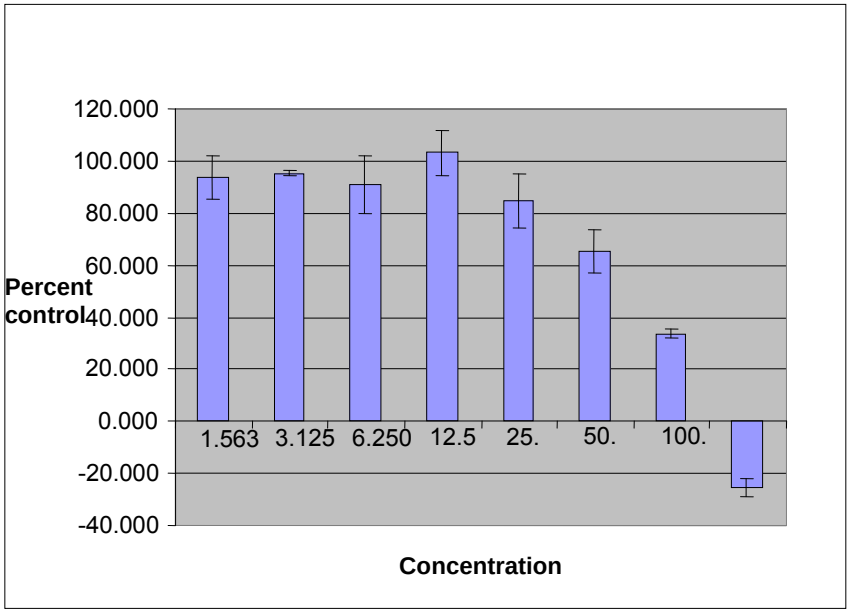
* IC_{50}: Fifty percent cytotoxic values ; [a] NT : Not tested; NA : Not applicable

The effect of crude extracts and pure compounds on *L. monocytogenes* biofilms was quantified following the method as stated by Amalaradjou *et al*. (2009). The confocal images of the control biofilm contain no added treatment (extracts or pure compounds) showed the formation of a dense biofilm (average thickness, 14 µm, maximum 30µm) (Figure 3.11 (a), while the images of

the treated samples (Compound **2** and **3**) exhibited breaks in the biofilm due to loss of cells and dismantling of organisation, as indicated by patches of few cells (Figures 3.11 (b) and 3.12 (b)).



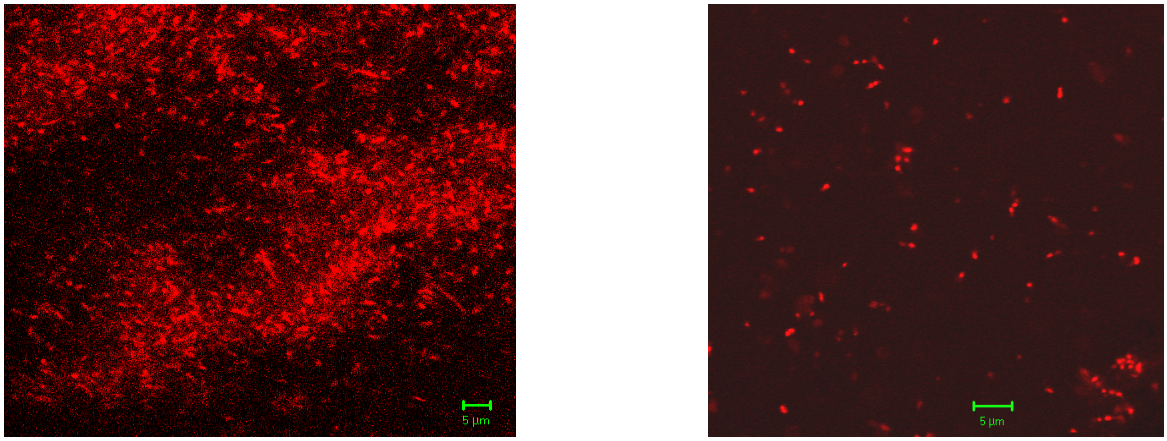
(a)



(b)

Figure 3.10 Dose response curve of (a) β-sitosterol (**2**), (b) epigallocatechin (**3**) compounds against Vero cell lines.

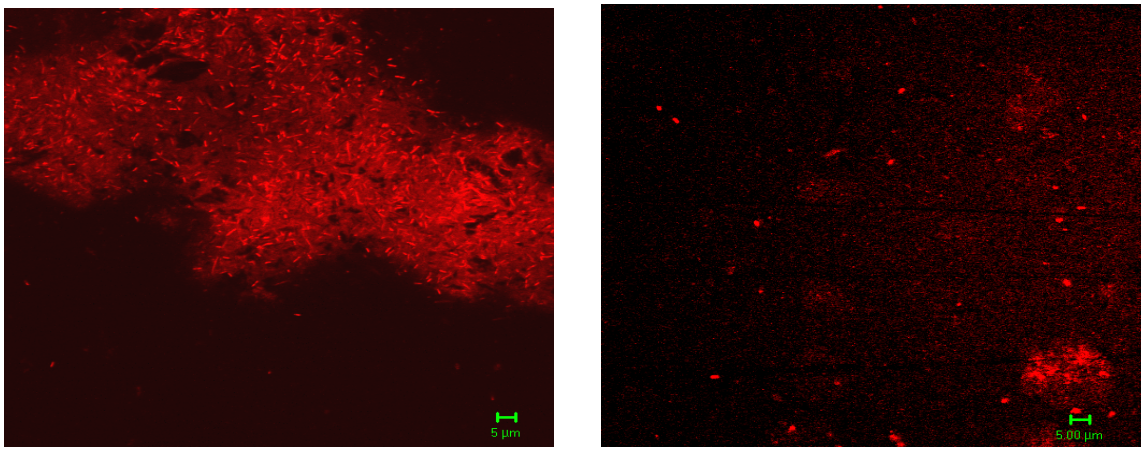
The crude extract showed a slight disruption of listerial biofilm (Figure 3.12 (a). The average thickness of the treated biofilms was 1 µm and the maximum thickness was 3 µm. These results are in agreement with the previous report who reported that the use of benzalkonium chloride, a quarternary ammonium compound reduced the formation of *L. monocytogenes* biofilms (Romanova *et al*., 2007). In the present study, β-sitosterol and epigallocatechin had good activity in the disruption of the *L. monocytogenes* biofilm. The aggregation of cells was disruption of cells which were exposed to was reduced from more than 25 µm in untreated cells to < 10 µm in diameter in the treated cells. *A. karroo* crude extract showed a slight disruption of listerial biofilm. Chi-Hua-Wu *et al*. (2008) reported the antibacterial effects of American cranberry (*Vaccinium macrocarpon*) concentrate on foodborne pathogen, *L. monocytogenes*.



(a) (b)

Figure 3.11. CSLM images of *L. monocytogenes* (LMG21263) biofilms without treatment (a) and after treatment with β-sitosterol (**2**). *L. monocytogenes* biofilm formed on cover slips were examined with Zeiss LSM 510 META confocal scanning laser microscope using a water immersion lens (scale: 1 unit represents 5 µm).

The results of transmission electron microscopy showed that the bacterial cell wall was damaged (Chi-Hua-Wu *et al*., 2008). The aggregation of cells which were exposed to β-sitosterol and epigallocatechin was reduced from 25µm as observed in untreated cells to < 10 µm in diameter in the treated cell.



(a) (b)

Figure 3.12 CSLM images of *L. monocytogenes* (LMG21263) biofilms after treatment with *A. karroo* (a) and after treatment with epigallocatechin (**3**). *L. monocytogenes* biofilm formed on coverslips were examined with Zeiss LSM 510 META confocal scanning laser microscope using a water immersion lens.

Generally biofilm microorganisms are resistant to antimicrobial agents than the same species in their planktonic form (Meyer, 2003). *L. monocytogenes* biofilms are difficult to remove even when normal cleaning routines are done on regular basis (Romanova *et al*, 2007). Both β-sitosterol and epigallocatechin could play a role as disinfectant agents and preliminary tests should be carried out to check their potential for removing listerial biofilm on contaminated surfaces.

A. karroo has not been tested on *L. monocytgenes* before. *A. karroo* has been tested on *S. aureus*, *E.coli*, *Agrobacterium tumefaciens*, *Erwina carotovora*, *Pseudomonas solanacearum*, *Clavibacter michiganense* and *Xanthomonas campestris* (Katerere and Eloff, 2003, Pretorius *et al*., 2003). *A. karroo* has been reported to be used medicinally for the treatment of diarrhoea (Appidi *et al*., 2008). Besides antibacterial activity, *A. karroo* has been reported to have antifungal activity (Pretorius *et al*., 2003; Kolaczkowski *et al*., 2009).

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Chapter 4

Isolation, characterisation and bioactivities of *Plectranthus ecklonii* constituents

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Isolation, characterisation and bioactivities of *Plectranthus ecklonii* constituents

Abstract

Plectranthus ecklonii Benth. is traditionally used in South Africa for treating stomach-aches, nausea, vomiting and meningitis. Bioassay-guided fractionation of *P.ecklonii* ethyl acetate extract led to the isolation of two known compounds, parvifloron D and parvifloron F. Both the isolated compounds, parvifloron D and F have not previously been reported as constituents of *P. ecklonii.* Parvifloron D and F exhibited minimum inhibitory concentration (MIC) of 15.6 and 31.25 µg/ml respectively against *L. monocytogenes.* The MICs of parvifloron D and F against a drug - sensitive strain of *Mycobacterium tuberculosis* were found to be 190 and 95 µg/ml respectively. The ethyl acetate extracts of *P. ecklonii* and its isolated compounds were tested for their activity on tyrosinase inhibition. The concentration of plant extract at which half the tyrosinase activity was inhibited (IC_{50}) was found to be 61.73 ± 2.69 µg/ml. The antibacterial activity of the extract of *P. ecklonii* and its isolated compounds correlates with the traditional use of the plant for various ailments such as stomach-aches, diarrhoea and skin diseases. The fifty percent inhibitory concentration of 'parvifloron D and 'parvifloron F' against vero cell lines were found to be 2.94 µg/ml and 1.56 µg/ml respectively. This is the first report on the bioactivities of extracts of *P. ecklonii* and its two constituents.

4.1 Introduction

Plant species belonging to the genus *Plectranthus* are found in Asia, Australia and Africa (Narukawa *et al*., 2001; Lukhoba *et al*., 2006). Several species of *Plectranthus* such as *P. barbatus, P. grandidentatus*, *P. hereroenes* have been reported to have antibacterial activity against *Staphylococcus aureus.* Diterpenes isolated from *P. grandidentus* and *P. hereroenes* were found to be active against methicillin resistant *S. aureus* (MRSA) (Matu & van Staden 2003; Gaspar-Marques *et al*., 2006). The leaves of *P. amboinicus* have been found to have antituberculosis activity (Narukawa *et al*., 2001)

Plectranthus ecklonii Benth. is traditionally used in South Africa for treating stomach-aches, nausea, vomiting and meningitis (Lukhoba *et al*., 2006) the symptoms associated with listeriosis infection. Leaves of the plants are also used for respiratory problems, chest complaints and coughs (TB- related problems) (Lukhoba *et al*., 2006). Aerial parts of the plant are used by people in Zimbabwe for skin diseases and skin hyper-pigmentation problems. *P. ecklonii* is widely distributed in South Africa, Australia, New Zealand, Mexico and the United States. *P. ecklonii* is a shrub which grows to about 2 metres tall. The plant grows best in semi-shade areas. The objectives of the present study were to scientifically validate the traditional uses of the plant and isolate the bioactive compounds.

4.2 Experimental: Materials and Methods

4.2.1 General experimental Procedures

^{1}H NMR and ^{13}C NMR spectra were recorded using a Brucker ARX 300 or a Brucker

Avance DRX 500 MHZ using $CDCl_3$, and DMSO-d_6 as solvents which led to the identification of the purified compounds.

4.2.2 Chemicals and Reagents

XTT (2,3- bis(2-methoxy-4-nitro-5-sulfophenyl)-5[(phenylamino) carbonyl]-2-H-tetrazolium hydroxide powder, DCFDA (2,7 dichloroflourescin diacetate), 2,4,6-triprydyl-s-triazine (TPTZ) and all chemicals reagents obtained from Sigma (UK). $FeCl_3.6H_2O$, sodium sulphate, $FeSO_4$, 2-thiobarbituric acid (TBA) were obtained from Merck (Germany). *L*-Tyrosine, *L*-DOPA, tyrosinase, arbutin and Kojic acid were obtained from Sigma-Aldrich (Kempton Park, South Africa). All chemicals and solvents were of the highest commercial grade.

4.2.3 Plant material

Leaves of *P. ecklonii* were collected from Pretoria, South Africa. Voucher specimens of *P. ecklonii* (PRU 96396) were identified and deposited at the H.G.W.J. Schweickerdt Herbarium (PRU), University of Pretoria, South Africa.

4.2.4 Isolation of the bioactive compounds from *P. ecklonii*

The leaves of *P. ecklonii* (550 g) were extracted with ethyl acetate (2 x 2L) for 48 hours, at room temperature (± 25°C). The ethyl acetate extract was filtered and concentrated to dryness using Rotavapor. The total extract (110 g) was subjected to silica gel column chromatography (CC, size 7 cm x 120 cm) using hexane/ethyl acetate mixtures of increasing polarity (0 to 100%)

(Figure 4.1). Similar fractions were combined into 5 main fractions based on the TLC profile (Figure 4.2). The five fractions were tested against *L. monocytogenes* and it was found that 2 fractions, fraction IV and V exhibited good antilisterial activity. Fraction V (2.9 g) was subjected to a Sephadex LH-20 column using dichloromethane/methanol mixtures (100 % dichloromethane, 99 %, 2 %, 3 %) (v/v) as an eluent to give compound P **1** (yield 2.31g, 0.42 %). Fraction IV (2.5 g), under the same conditions gave pure compound P **2** (yield 937 mg, 0.17 %).

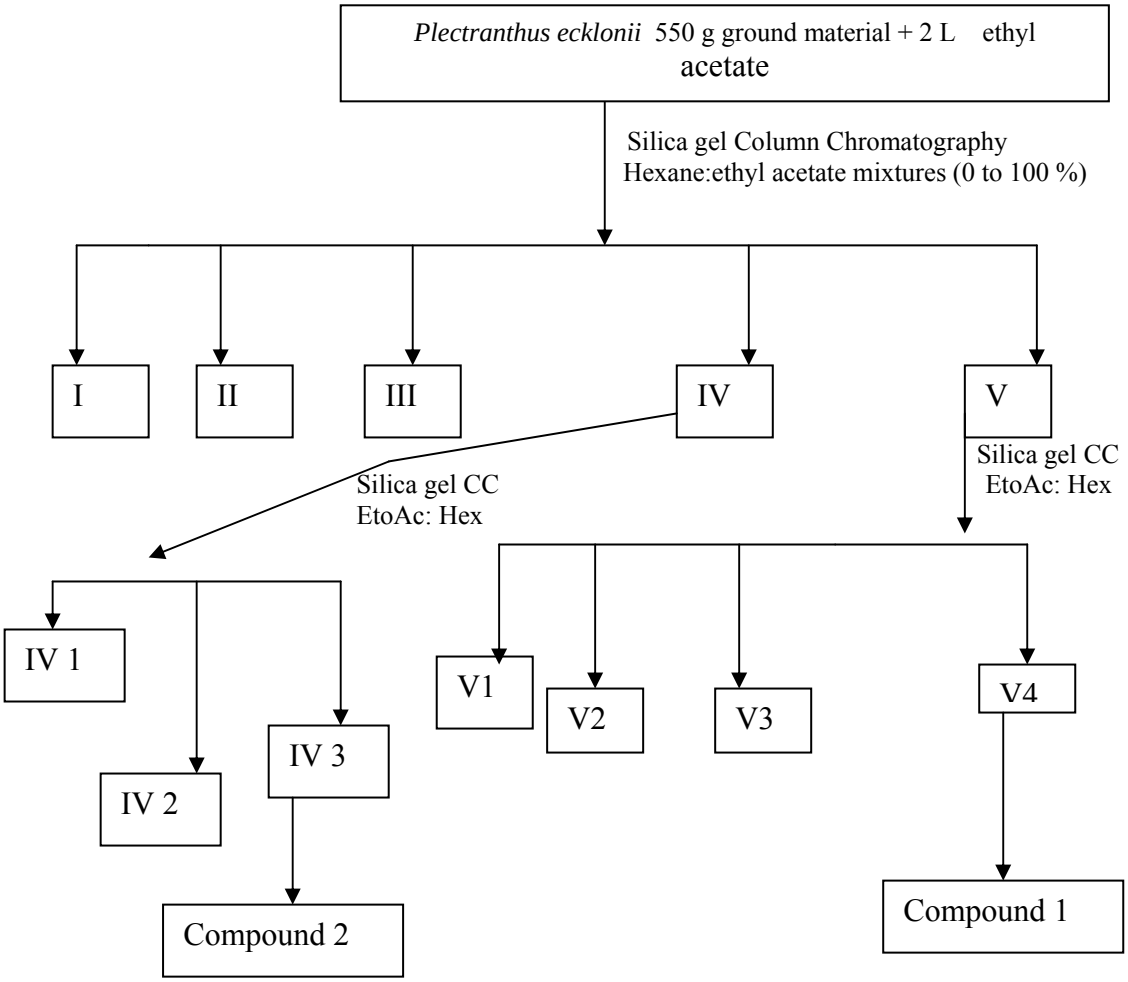
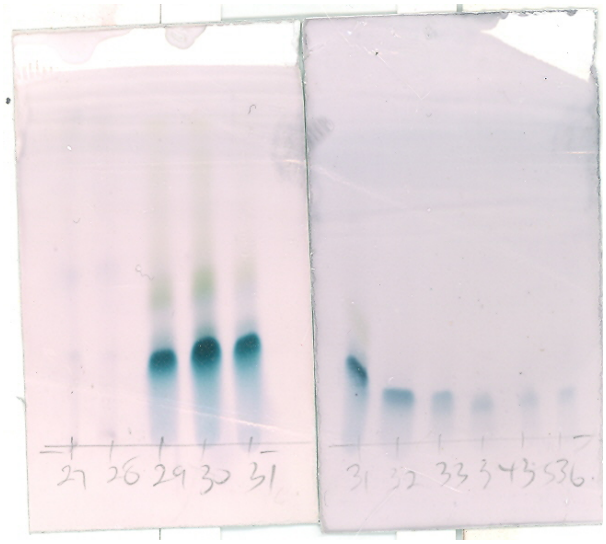


Figure 4.1 Schematic representation of the isolation process of the bioactive compounds from *Plectranthus ecklonii*

(a)



(b)

(c)

Figure 4.2 (a) Silica gel column chromatography of the ethyl acetate extracts of *P. ecklonii*, (b) fractions obtained by column chromatographic purification, spotted onto the TLC plates and (c) direct bioassay on TLC plates.

4.2.5 Activity of samples against *Listeria monocytogenes* and other bacteria

The bacterial culture of *L. monocytogenes* (LMG 21263) was activated by transferring a loop full from brain heart infusion (BHI) slants into tryptone soya broth (TSB) followed by incubation at 37 °C for 24 hours (Alzoreky & Nakahara, 2003). The optical density of the culture was adjusted to 0.1 at 590 nm using fresh broth to give a standard inoculum of 10^6 colony forming units (CFU/ml). The bacterial count was confirmed by plating out on TSA and Mueller–Hinton agar plates incubated at 37 °C for 24 hours. Stock cultures were maintained at -70 °C (Alzoreky & Nakahara, 2003). The minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) determination were done as stated previously (Eloff, 1998; Mathabe *et al*., 2008). Briefly, the crude extracts and purified compounds were dissolved in 2.5% DMSO to obtain a stock solution. The DMSO (2.5%) was used as control to test the inhibition of the bacteria. The concentrations of the crude extract and the compounds tested ranged from 7.81 to 1000 µg/ml. Double fold serial dilutions of each sample was performed. Tests were done in triplicate. The plates were sealed and incubated at 37 ºC for 24 hours. The MIC of the samples was determined by adding 40 µl of (0.2 mg/ml) *p*-iodonitrotetrazolium violet (INT) (Sigma-Aldrich, South Africa) to microtitre wells and the re-incubated at 37 ºC for 1 hour (Eloff, 1998; Mativandlela *et a*l., 2006) to indicate the presence of bacterial growth (pink colour) or inhibition of bacterial growth (no colour). The MIC was defined as the lowest concentration of the extract that caused no colour change and showed complete inhibition of bacterial growth. The minimum bactericidal concentration (MBC) was determined by transferring 50 µl of the sample from the wells which did not show bacterial growth during the MIC assays (without INT), to 150 µl of the freshly prepared broth. The plates were re-incubated at 37 ºC for 48 hours. The MBC was regarded as the lowest concentration which did not give rise to a colour change

after INT was added as described above in the MIC assay. Erythromycin was used as a drug control for *L. monocytogenes.*

Culture of *Staphylococcus aureus* (ATCC 12600), *Escherichia coli* (ATCC 25922), *Enterococcus faecalis* (ATCC 29212), and *Pseudomonas aeruginosa* (ATTCC 27853) were each transferred on to nutrient agar slant and the culture was then recovered for testing by growing on a nutrient broth (BIOLAB, South Africa) for 24 hours at 37 ºC . Ciprofloxacin was used as a drug control. The MIC and MBC was determined as stated above.

4.2.6 Antimycobacterial bioassay

Mycobacterium smegmatis (MC^2 155) and a drug susceptible strain of *M. tuberculosis* H37Rv (ATCC 27264) were acquired from *American* Type, MD, USA Culture Collection. The microplate dilution method was used for the testing of the samples against *M. smegmatis* in 96-Well microtitre plates according to Salie *et al*. (1996). The susceptibility testing of *M. tuberculosis* was done with the radiometric respiratory technique using the BACTEC 460 system (Becton Dickinson Diagnostic Instrument, Sparks, MD) as described previously (Lall & Meyer,1999; Lall & Meyer, 2000; Lall & Meyer, 2001). The crude extracts and purified compounds were dissolved in 2.5% DMSO in sterile 7H9 broth to obtain a stock concentration of 1.250 mg/ml. The DMSO (2.5%) was used as control to test the inhibition of bacteria. The final concentration ranged from 9.76-312.50 µg/ml.

4.2.7 Antityrosinase assay

Melanin is a key pigment responsible for skin and / or hair colour. Its production is

catalysed by an enzyme called 'tyrosinase'. Since, traditionally the paste made of the leaves of *P. ecklonii* are used by South Africans for skin-hyperpigmentation problem, it was therefore, decided to test the extract and purified compounds of *P. ecklonii* for their activity on tyrosinase inhibition. The method as previously described (Nerya *et al*., 2003) was followed with modifications. The drug control used was the kojic acid (Kim *et al*., 2004). Briefly each sample (crude extract or compound) was dissolved in DMSO to a final concentration of 20 mg/ml. The final percentage of DMSO was 1.5% for both crude extract and pure compound. The sample stock solution was then diluted to 200 µg/ml in a 50 mM potassium sulphate buffer. Seventy microlitre of each sample dilution was combined with 30 µl of tyrosinase (333 units/ml in phosphate buffer) (This was done in triplicate) in a 96-well microtitre plates. After the elapse of five minutes 110 µl of the substrate (2 mM L-tyrosinase or L-DOPA) was added to each well. The final concentrations of the samples ranged from 200 to 12.5 µg/ml. The plates were then incubated at room temperature for 30 min. Optical densities of the reaction mixtures in the wells were then recorded at 492 nm with the BIO-TEK power Wave XS multi well plate reader.

4.2.8 Cytotoxicity test

The method as stated in section 3.2.4 of this thesis was followed to determine the the cytotoxicity test.

4.2.9 Effect of the crude extract of *P. ecklonii* and its compounds on listerial biofilms

The method as stated in section 3.2.4 of this thesis was followed to determine the listerial biofilm formation on exposure to the purified compounds.

4.3 Results and Discussion

The two known compounds were isolated from ethyl acetate extract of *P. ecklonii*. The respective NMR spectra are as follows:

Compound P **1**

The compound P **1** was isolated as orange crystals from the non polar part of the extract of *P. ecklonii*. The structure of compound P **1** was established to be parvifloron D based on the spectroscopic data (Figure 4.1). ^{1}H NMR signals at 6.40 (d, J = 6.8; H - 6); 6.75 (d, J = 6.8, H – 7); 6.94 (d, J =0.5, H-14); 5.59 (s, H-2β), five methyl groups signals at 1.15, 1.19 (each d, J =6.4, Me-16,17) 1.29 (s, Me-18), 1.41 (s, Me-19), 1.69 (s, Me–20). The ^{13}C- NMR indicated the presence of 27 carbons, 20 of which of the diterpene skeleton and the rest represent the signals of *p*-benzoic acid derivatives (Figure 4.3). The signal of 2β was shifted to low field due to the esterification of the *p*-benzoic acid with the OH at the same position. This was confirmed by the comparison of the obtained data with those of published in literature. Compound P**1** is a terpene. Compound P **1** (parvifloron D) was previously isolated from *P. strigosus* (Gaspar-Marques *et al*., 2008). Compound P **1** has been reported to have antibacterial activity against *Staphylococcus* and *Enterococcus* species, including methicillin- and vancomycin-resistant strains (Simões *et al*., in press). Compound P **1** has not been tested on *L. monocytogenes*.

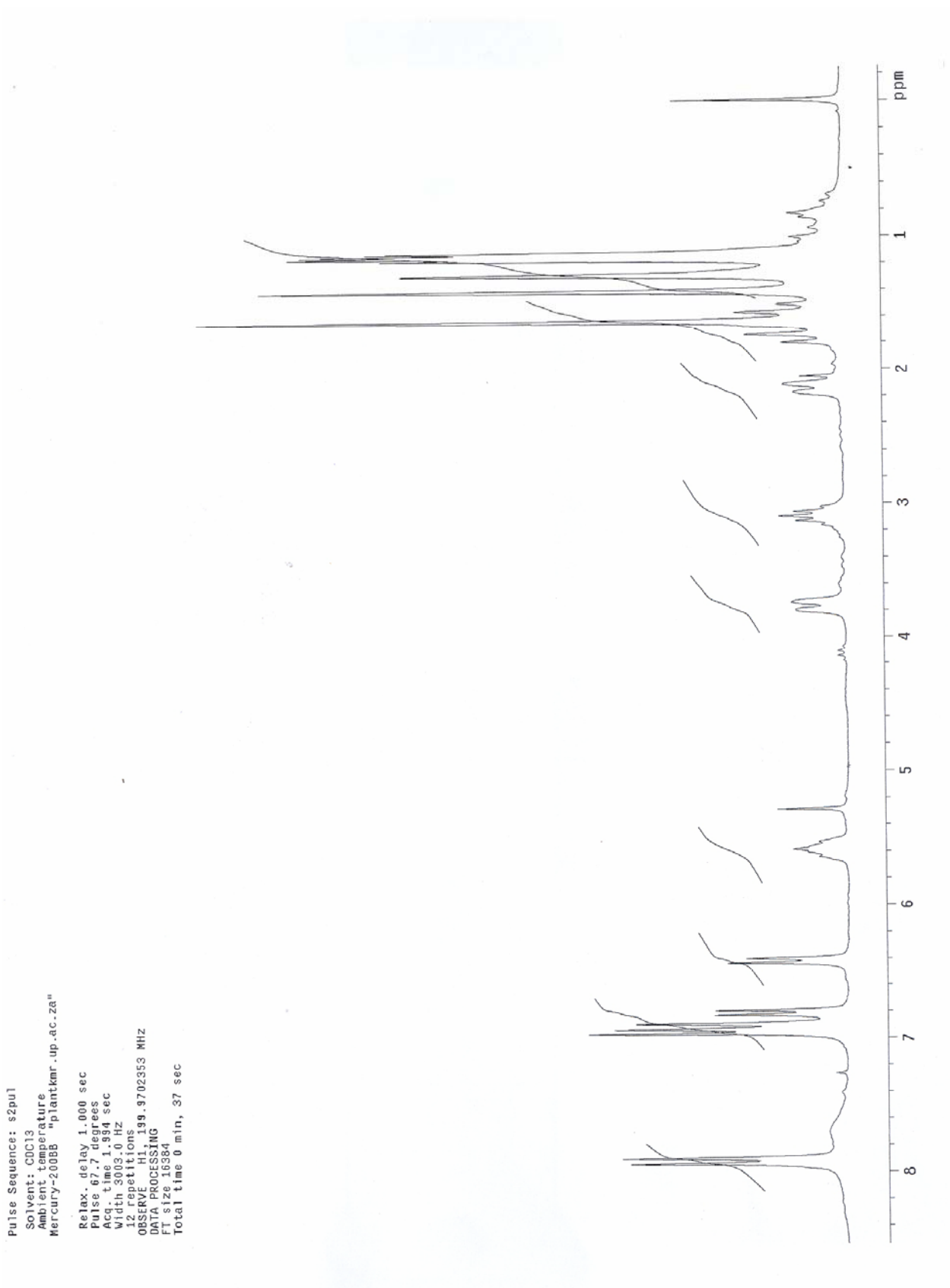


Figure 4.3 ^{1}HNMR sectrum of compound P **1** (parvifloron D) isolated from *P. ecklonii*

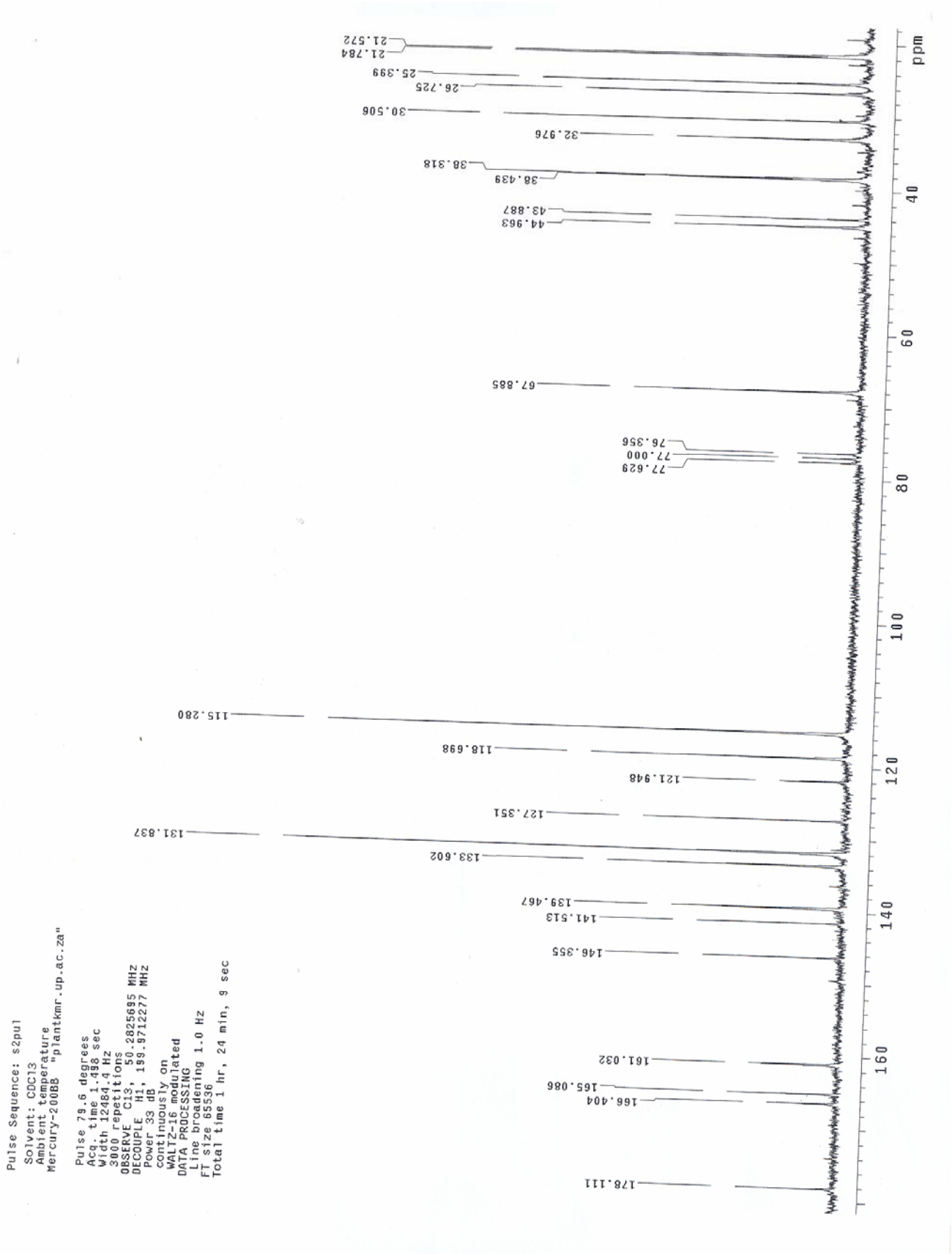


Figure 4.4 ^{13}CNMR spectrum of compound P **1** (parvifloron D) isolated from *P. ecklonii*

Compound P **2**

Compound P **2** (parvifloron F) showed NMR data (Figures 4.3 and 4.4) similar to those of compound P **1** (parvifloron D) except for the side chain, which showed signals of 1,3,4-trisubstituted benzoic acid patterns instead of 1,4-disubstituted benzoic acid. The data obtained also were compared with those, which published in literature. Compound P **2** belongs to the diterpenes group of compounds. Compound P **2** (parvifloron F) was previously isolated from *P. nummularius* (Narukawa *et al*., 2001). Not much has been reported in literature about the antibacterial activity of compound P **2** except its anti-oxidative activity (Narukawa *et al*., 2001). Compound P **2** has not been tested on *L. monocytogenes*. Both the isolated compounds, Parvifloron D and F have not previously been reported as constituents of *P. ecklonii*. Figure 4.5 shows the chemical structures of compounds P **1** and P **2.**

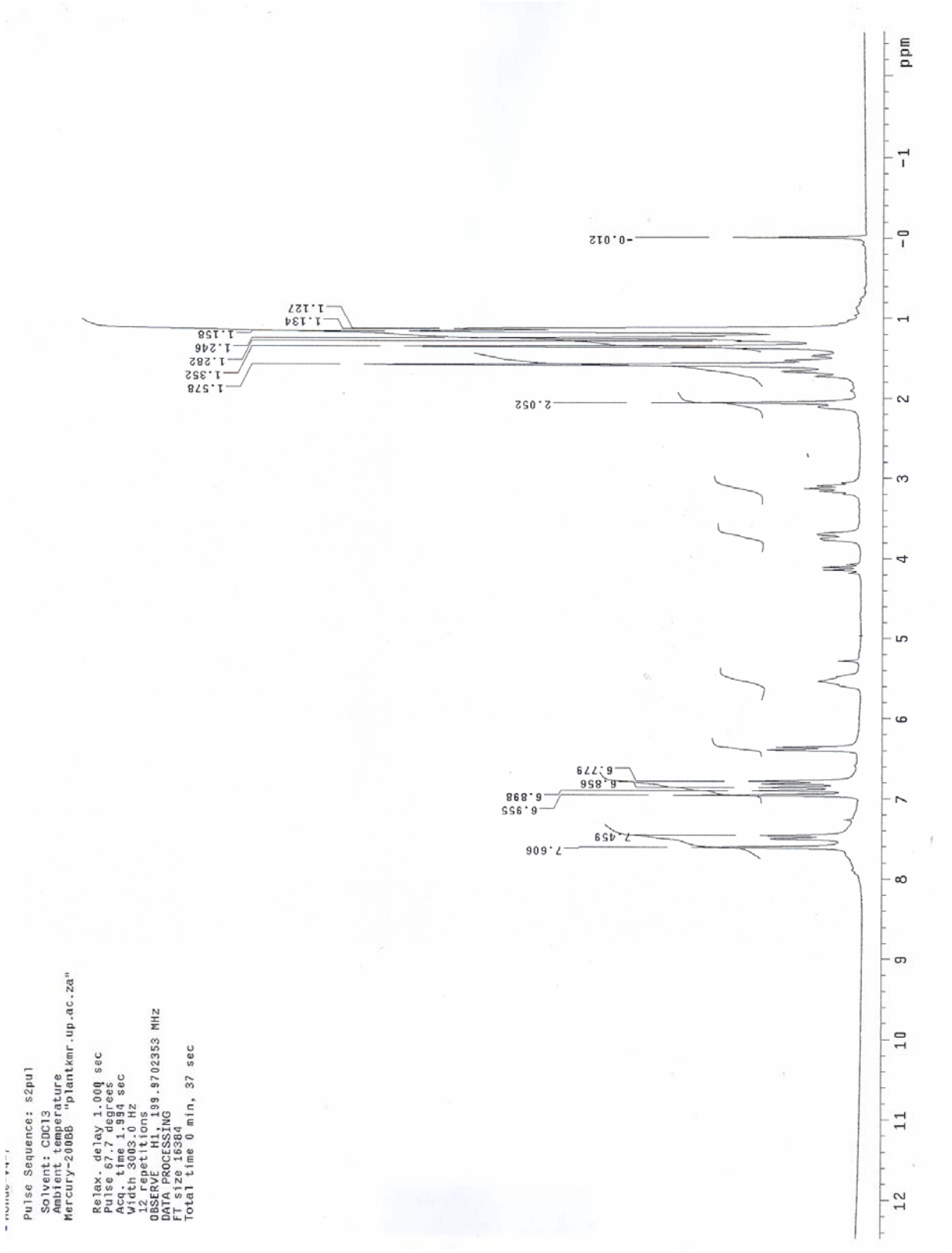


Figure 4.5 [1]HNMR spectrum of compound P **2** (parvifloron F) isolated from *P. ecklonii*

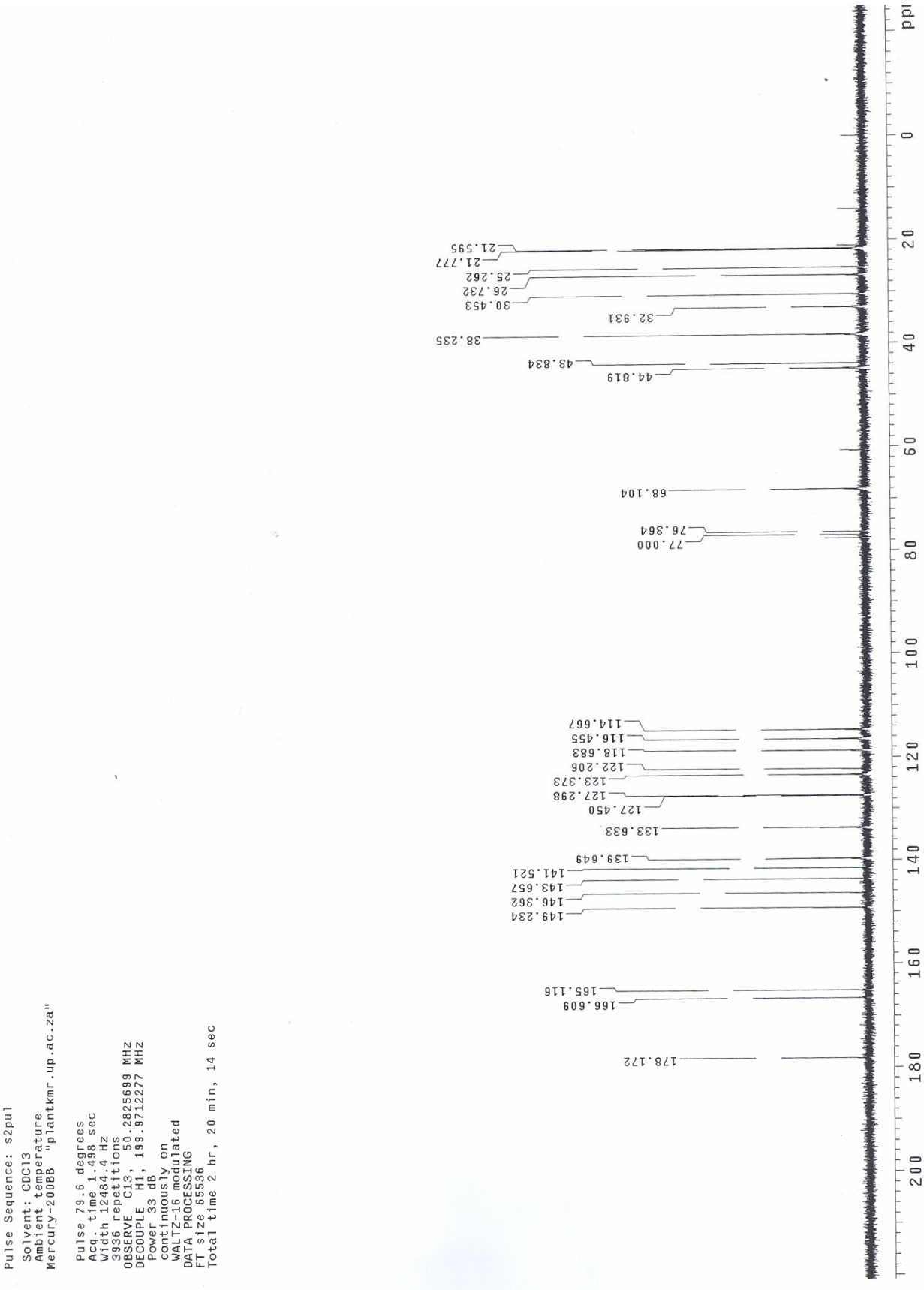
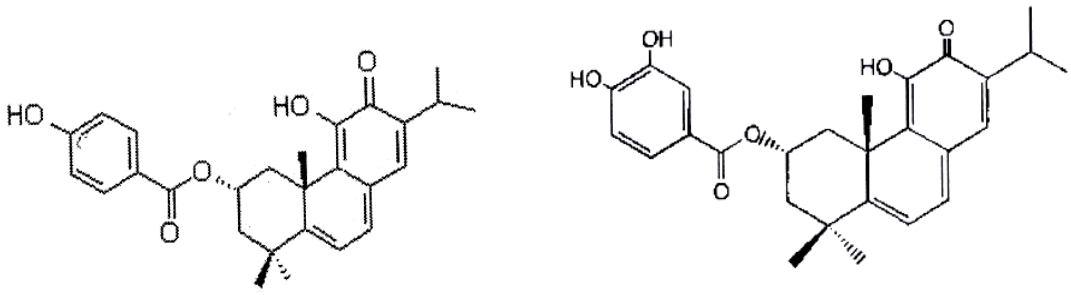


Figure 4.6 ^{13}C NMR spectrum of compound P **2** (parvifloron F) isolated from *P. ecklonii*



P **1** P **2**

Figure 4.7 Chemical structures of P (**1**) parvifloron D and P (**2**) parvifloron F

The ethyl acetate extracts of the leaves of *P. ecklonii* showed good activity against *Listeria monocytogenes* (LMG 21263) exhibiting a minimum inhibitory concentration (MIC) of 500 µg/ml. In previous studies the MIC of other plants have been found to be generally higher than the one observed in this study. Alzoreky and Nakahara (2003) reported the MIC values of methanolic extract of *Artemisia absithium* and eighteen other plants as ranging from 1.32 mg/ml to 2.64 mg/ml against *L. monocytogenes*.

Column chromatographic purification of the ethyl acetate extracts of the aerial parts of *P. ecklonii* resulted in five major fractions. The five fractions were tested against *L. monocytogenes* and it was found that 2 fractions, fraction IV and V exhibited a MIC of 62.5 µg/ml and 125 µg/ml respectively. Fractions I – III were not found to be active against *L. monocytogenes* at the highest concentration (500 µg/ml) tested. The two known compounds that were isolated from *P. ecklonii* were compound **1** (from fraction IV) and compound **2** (from fraction V)

Other compounds that have been previously isolated from *P. ecklonii* are 'ekclonoquinone A', 'ecklonoquinone B', 4',5-dihydroxy-6,7-dimethoxyflavanone, nepetoidin A and B (Grayer *et al*., 2003; DNP, 2010) Nepetoidin A and B have been reported to exhibit antifungal activity against *Aspergillus niger* (Grayer *et al*., 2003).

Besides the above data, not much is known about phytochemical properties of this plant. The MICs of the compounds P **1** and P **2** against *L. monocytogenes* were found to be 15.60 µg/ml and 31.25 µg/ml respectively. These results are comparable to the MICs of 125 µg/ml obtained for both (*Heracleum sphondylium* subsp. *ternatum*) and its main component, 1-octanol against *L. monocytogenes* (İscan *et al*., 2003).

The MICs of *P. ecklonii* ethyl acetate extracts against Gram-positive and Gram-negative bacteria species ranged from 125.0 to 1000.0 µg/ml (Table 4.1).

Compounds purified from *P. ecklonii* ethyl acetate extracts were found to be active at a low concentration of 31.3 µg/ml against some of the bacterial species tested. The MIC of other *Plectranthus* species, such *P. cylindraceus* oil against *E. coli* was found to be 125 µg/ml (Marwah *et al*., 2007) which is similar to the results obtained in the present study. In the previous study it has been reported that *E. globulus* was found to have antibacterial activity against as *S. aureus* and *E. coli* (Välimaa *et al*., 2007). .*P ecklonii* is traditionally used to treat skin infections in Zimbabwe (Lukhoba *et al*., 2006). The traditional use of *P. ecklonii* for skin infections could be possibly linked to the antibacterial activity of parvifloron D (P **1**) and parvifloron F (P **2**) against *S. aureus*. *Plectranthus* species have been traditionally used in folk medicine to treat digestive and stomach-ache (Lukhoba *et al*., 2006). The activity of *P. ecklonii* against *Escherichia coli* observed in the present study justifies the use of *Plectranthus* species in traditional medicine in the treatment of gastro-enteric infections.

Table 4.1: Antibacterial and antityrosinase activity of the ethyl acetate extracts of the aerial parts of *Plectranthus ecklonii* and its isolated compounds. Cytotoxicity of the extract and the isolated compounds against Vero monkey cells.

Bacteria tested	**MIC / MBC [a] (µg/ml)**						
	Ethyl acetate extract *of P. ecklonii*	Compound **1** (parvifloron D*)*	Compound **2** (parvifloron F)	RA[b]	Kojic acid[c]	Doxorubicin[d]	Isoniazid[e]
E. coli	125.0 500.0	31.25 31.25	31.25 62.5	1.2 1.2			
E. faecalis	250.0 250.0	31.25 62.5	62.5 62.5	18.75 18.75			
L. monocytogenes	500.0 1000.0	15.6 31.25	31.25 62.5	1.2 1.2			
S. aureus	250.0 250.0	31.25 31.25	15.6 31.25	9.4 9.4			
P. aeruginosa	250.0 250.0	31.25 31.25	31.25 62.5	18.75 18.75			
M. smegmatis	78.12 625.0	39.06 156.25	39.06 78.1	0.61 1.22			
M. tuberculosis	380.0 >380.0	190.0 190.0	95.0 190.0				0.12 0.12
IC_{50} (Antityrosinase activity	61.73 ± 2.69	Na[g]	Na		2.145± 0.08		
IC_{50} (cytotoxicity testing)	< 3.1	2.935	< 1.56			0.5449	
SI[f] (IC_{50}/MIC)	0.06	0.19	0.05	8.46			

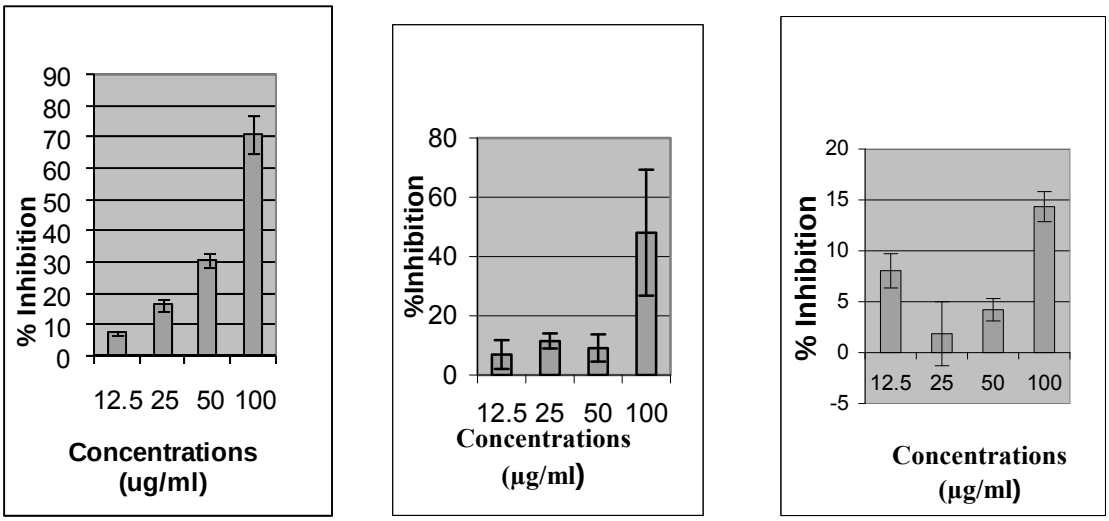
[a]MIC: Minimum inhibitory concentration; [b]RA: Reference antibiotics: erythromycin (positive drug control for *Listeria monocytogenes*), ciprofloxacin (positive drug control for *Mycobacterium smegmatis, Escherichia coli*, *Enterococcus faecalis*, *Staphylococcus aureus*, and *Pseudomonas. aeruginosa*); [c]Kojic acid (positive drug control for antityrosinase bioassay); [d]Doxorubicin (positive drug control for cytotoxicity testing); [e]Isoniazid (positive drug control for *Mycobacterium. tuberculosis*); [f]SI: (IC_{50}/MIC) Selectivity index;

[g]Na : not active at the high concentration (100 µg/ml) tested.

The anti-mycobacterial activity of the investigated crude extract of *P. ecklonii* and its isolated compounds are shown in Table 4.1 Both compounds P **1** and P **2** showed good activities against *M. tuberculosis*. The MIC of 95 µg/ml exhibited by compound **2** in the present study was similar to the reports on the inhibitory activity of shinanolone and mamegakinone against *Mycobacterium tuberculosis* H37RV strain (Van der Kooy *et al*., 2006). The MIC of the *P. ecklonii* crude extract against *M. tuberculosis* H37RV strain (380 µg/ml) was found to be comparable to those of previous studies on a number of *Helichrysum* species that exhibited MICs of 0.5 mg/ml (500.0 µg/ml) (Marwah *et al*., 2007). A number of other plants such as *Pelargonium reniforme*, *P. sidoides*, *Achillea millefolium*, *Ageratum corimbosum*, and *Anoda cristata* have been reported to have MIC values ≥ 1.0 mg/ml against *M. tuberculosi* (Jiminez-Arellanes *et al*., 2003; Mativandlela *et al*., 2006). The crude extract of *P. ecklonii* exhibited an MIC of 78.12 µg/ml against a non pathogenic *Mycobacterium* species i.e., *M. smegmatis*. Our results are in agreement with previous findings where it was stated that extracts of *Mentha piperita*, *Cinnamomum zeylanicum* gave an MIC of 500.0 µg/ml against *M. smegmatis* (Newton *et al*., 2002).

Both compounds P**1** and P **2** also showed good anti-mycobacterial activity against *M. smegmatis* at a concentration of 39.06 µg/ml (MIC). The selectivity index (SI = IC_{50}/MIC) calculated to 0.1 and 0.05 for parvifloron D (P **1**) and parvifloron F (P **2**) respectively. Parvifloron D (P **1**) had the highest SI value compared to parvifloron F (P **2**) and the *P. ecklonii* ethyl acetate crude extract in the present study. This is in accordance with the literature (Kovala-Demertzi *et al*., 2009) where the compound with the highest actvity was also found to be more selective (high SI value) than the compound with less activity.

The ethyl acetate extract of *P. ecklonii* and its isolated compounds were tested for their activity on tyrosinase inhibition (Figure 4.8). The concentration of plant extract at which half the tyrosinase activity was inhibited (IC_{50}) was 61.73 ± 2.69 µg/ml.



(a) (b) (c)

Figure 4.8 Dose response curve of antityrosinase activity of (a) *P. ecklonii* crude extracts (b) parvifloron D (P **1**), (c) parvifloron F (P **2**)

During cytotoxicity evaluation (Figures 4.9 and 4.10) the fifty percent inhibitory concentration of 'parvifloron D and 'parvifloron F' against vero cell lines were found to be 2.94 µg/ml and 1.56µg/ml respectively (Table 4.1).

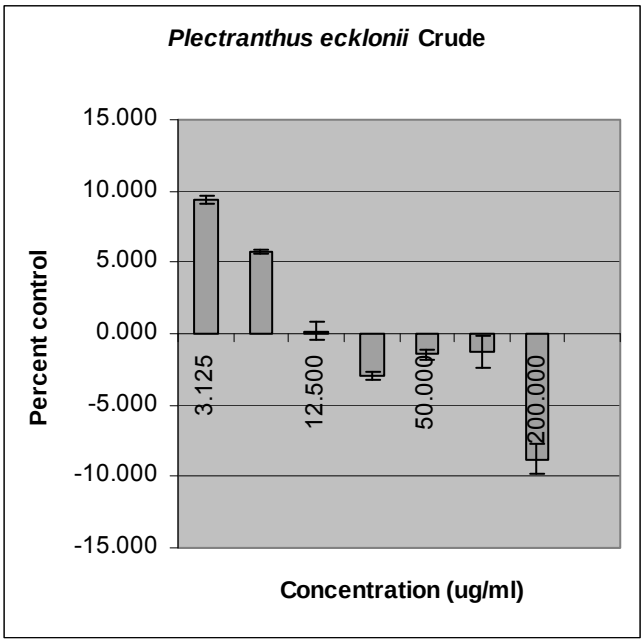
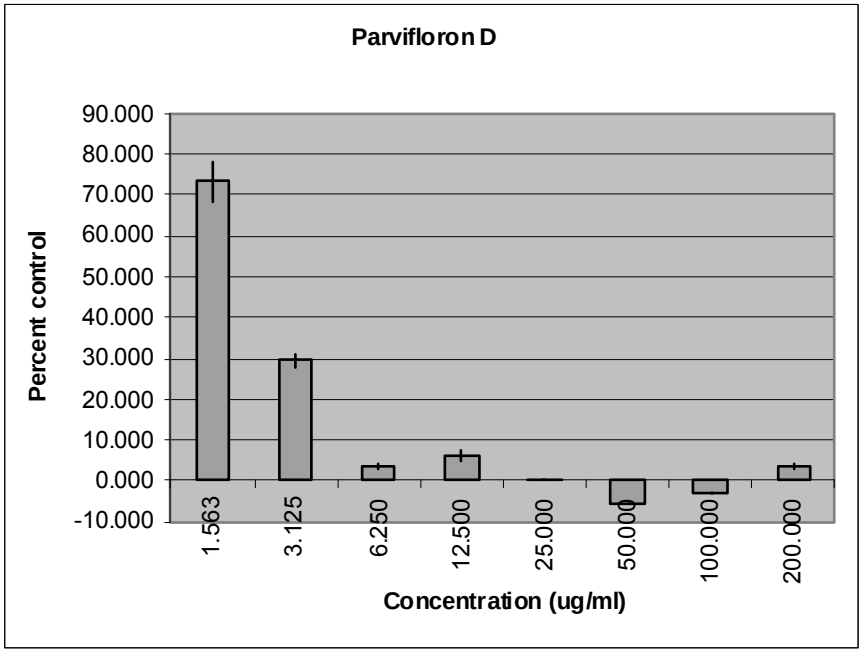


Figure 4.9 Dose response curve of (a) *P. ecklonii* ethyl acetate extract against Vero cell lines.

Results of the cytotoxicity tests showed that isolated compounds were toxic as shown by the fifty percent inhibing concentration (IC_{50} values) against monkey kidney Vero cell lines (Table 4.1). From the graphs that shows cytotoxicty, the viability cells for *P. ecklonii* crude and pure compound, parvifloron F (P **2**) the Vero cells viability never reached up to 50%. This has had an impact on the calculation of the IC_{50} values .The reason could be that the the extract and the compound were too toxic to the cells. The negative values from the graph are due to the colouraton of the extracts. .It has been reported that compounds with the lowest minimal inhibitory concentrations (MIC) are also the most cytotoxic against green monkey Vero cell line (Zentz *et al*., 2004). This is in contrast with literature were compounds with lower MICs showed least cytotoxicity against the Vero cells (Mathabe *et al*., 2008). The antibacterial activity of the extract of *P. ecklonii* and its isolated compounds correlates with the traditional use of the plant for various ailments such as stomacha-ches, diarrhoea and skin diseases.



(a)

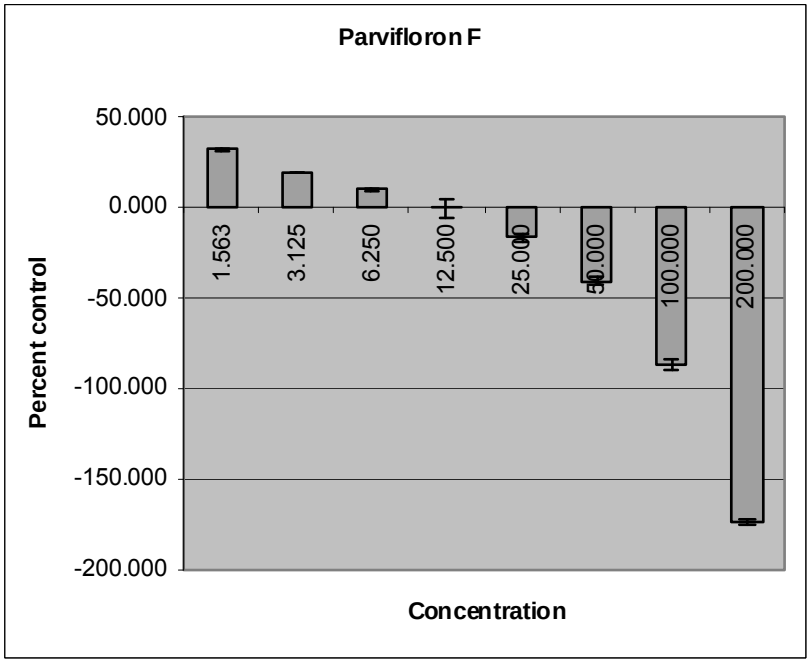
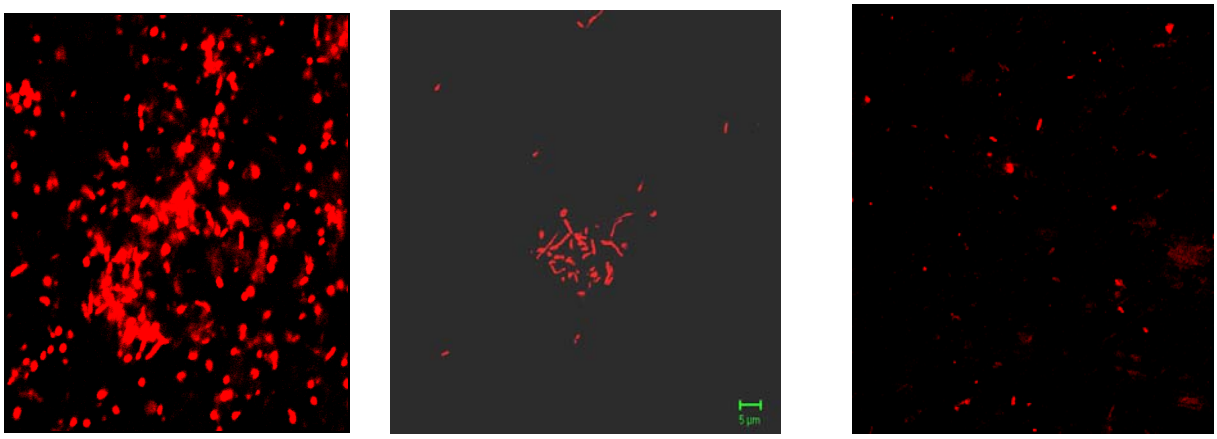


Figure 4.10 Dose response curve of (a) parvifloron D (P **1**), (b) parvifloron F (P **2**) against Vero cell line

The untreated listerial cells formed a dense biofilm while the cells treated with parvifloron D (P **1**) disrupted the formation of a listerial biofilm (Figure 4.11 (c)). In a previous study it was reported that the use of American cranberry (*Vaccinium macrocarpon*) showed the activity against the foodborne pathogen, *L. monocytogenes* (Chi-Hua Wu, *et al*, 2008).

Figure 4.11 (b) showed a slight reduction in the development of the listerial biofilm. In this study both parvifloron D (P **1**) and parvifloron F (P **2**) had greater activity in the disruption of the *L. monocytogenes* biofilm. The aggregation of cells which were exposed to parvifloron D (P **1**) and parvifloron F (P **2**) was reduced from 25µm as observed in untreated cells to < 10 µm in diameter in treated cells.



(a) (b) (c)

Figure 4.11 CSLM images of *L. monocytogenes* (LMG21263) biofilms without treatment (negative control) (a), (b) after treatment with *P. ecklonii* and (c) after treatment compound, 'parvifloron D' (P **1**). *L. monocytogenes* biofilm formed on coverslips were examined with Zeiss LSM 510 META confocal scanning laser microscope using a water immersion lens.

In the previous study, knotwood and bark extracts of *Picea maritma* and *Eucalyptus globulus* respectively, were found to have antibacterial activity against *L. monocytogenes*. The flavonoid, pinocembrin has been previously reported to be active against *L. monoctogenes* (Välimaa *et al*., 2007). In another study it has been previously reported that cranberry, cloudberry, raspberry and strawberry have shown activity against *L. monocytogenes* (Puupponen-Pimiä *et al*., 2005).

P. ecklonii has not been tested before on *L. monocytogenes*. *P. ecklonii* has been tested on *Streptococcus sobrinus*, *S. mutans*, *Staphylococcus aureus*, *S. epidermis*, *Bacillus subtilis* and *Micrococcus luteus* (Rabe and Van Staden, 1998; Figueiredo *et al*., 2010). *P. ecklonii* has been reported to be medicinally used for skin problems in South Africa (Lukhoba *et al*., 2006).

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CHAPTER 5

General Discussion and Conclusion

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General Discussion and Conclusion

5.1 Motivation of this study

Foodborne bacterial illnesses are still a major health concern in both the developing and developed countries (Alzoreky & Nakahara, 2003). *Listeria monocytogenes* has been implicated as the culprit to most deaths caused by food related illnesses (White *et al.,* 2002). Plants have been used in folk medicine against symptoms caused by listeriosis. Furthermore, this organism has been found to be resistant to several single antibiotics (Nichterlein *et al*., 1998; White *et al*., 2002) and results in the need for multidrug therapy (Cone *et al*., 2003). The use of multidrug treatment however results in side effects (Gleckman and Borrego, 1997) and this has led to the use of medicinal plants as an alternative treatment for listeriosios.

Various medicinal plants have been used in daily life to treat diseases all over the world (Ates and Erdogrul, 2003). It is through the development of traditional medicine that the therapeutic effect of drugs has been revealed (Hikino, 1991). The use of medicinal plants by South Africans in treating symptoms associated with listerioisis such as, diarrhoea, headaches, fever, inflammation, prevention of abortion etc has been documented (Van Wyk *et a*l. 1997). The problem of multidrug resistance has necessitated the need to explore the potential of South African medicinal plants for antilisterial activity. Finding the most effective novel drugs from plants against *L. monocytogenes* could reduce the risk of multidrug resistant species and reduce the treatment costs. Scientific basis for the use of such plants in South African traditional medicine was required and therefore, against this background the antilisterial activity of 13

local medicinal plants were investigated. The plant extracts were screened for activity against *Listeria monocytogenes.*

5.2 Discussion and conclusion

Most of the studies of antimicrobial activity of plants against *L. monocytogenes* have focused on the planktonic cells. In the present study both planktonic cells and biofilm with regard to the bioactivity of plants against *L. monocytogenes* were investigated. The results obtained from the present study showed the potential of crude plants extracts of *A. karroo* and *P. ecklonii* together with their isolated pure compounds in combating listeriosis. Quave *et al*. (2008) have reported that biofilms are associated with severe infections. Listerial biofilms fall in this category of pathogens.

From the 13 plants that were screened for antililsterial activity, two plants *A. karroo* and *P. ecklonii* showed more activity as compared to the other eleven plants. This necessitated further investigation of the two plants. As stated in Chapter 2 the two plants have not been previously tested against *L. monocytogenes.*

From the three known compounds isolated from *A. karroo*, epicatechin (**1**) did not exhibit activity against *L. monocytogenes*. β-sitosterol (compound **2**) and epigallocatechin (**3**) showed activity against the pathogen. Although epicatechin (**1**) did not show activity against *L. monocytogenes*, in the previous study it has been reported to have anti-inflammatory activity. In literature it has been reported that components of Chinese green tea extract such as epicatechin, epigallocatechin gallate, epicatechin gallate and caffeine when acting together were able to show activity against *L. monocytogenes* and other major food-borne pathogens, however when they were tested on its own, not in combination they did not show activity against the *L. monocytogenes* (Si *et al*. 2006). In the present study epigallocatechin (**3**) which is similar to

the previous mentioned compounds not only showed activity against the planktonic cells of *L. monocyo*genes but also against the listerial biofilm. Bacterial biofilm in particular listerial biofilm are more resistant to action of antimicrobial agents (García-Almendárez *et al*., 2007), however in this study the β-sitosterol (**2**) also showed good activity against *L. monocytogenes* biofilm. β-sitosterol (**2**) has been reported in literature to have antibacterial activity against Gram positive bacteria such as *S. aureus* and *B. subtilis* (Sanches *et al*., 2005). *L. monocytogenes* is a Gram positive bacteria like the other two previous mentioned organisms. Besides showing the lowest value, β-sitosterol (**2**) also exhibited moderate (IC_{50}) value as compared to the control drug during cytotoxicty test.

P. ecklonii and its two isolated compounds showed activity against *L. monocytogenes*. Parviflon D (compound P**1**) and parvifloron F (compound P**2**) not only showed activity against *L. monocytogenes* but also against a variety of organisms such as *Mycobacterium tuberculosis, M. smegmatis, S. aureus, Pseudomonas aeruginosa, E. coli* and *Enterococcus faecalis.* This is the first report on the bioactivity of the two compounds against *L. monocytogenes* (Nyila *et al*., 2009). The activity of *P. ecklonii crude* in the tyrosinase assay and also its antibacterial activity against *S. aureus* in the present study clearly demonstrate as to why the plant is used for skin related ailments (Lukhoba *et al*., 2006). The results of the present study illustrate that the two active compounds isolated from *A. karroo* and the two from *P. ecklonii* could play role as disinfectant agents and preliminary tests should be carried out to check out their potential for removing listerial biofilm on contaminated surfaces.

5.7 References

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CHAPTER 6

ACKNOWLEDGEMENTS

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APPENDICES

APPENDIX 1- Presentation at conferences

The work resulting from this thesis was presented at the following conferences

Local conference

Monde Nyila, Namrita Lall, Marion Meyer, Ahmed Hussein, Carmen Leonard & Boet Weyers. Susceptibility of *Listeria monocytogenes* to South African plants.

Indigenous Plant Use Forum (IPUF). 2-5 July 2007, University of Johannesburg, South Africa.

International conference

Nyila M.A., Lall N, Leonard C, Weyer B & Meyer J.J.M. Susceptibility testing of *Listeria monocytogenes* using the disc method and micro titre plate (micro broth dilution) method. 55th International Congress & Annual Meeting of the Society for Medicinal Plant Research. 2-6 September 2007. Graz, Austria.

APPENDIX 2- Publications

MONDE A. NYILA, CARMEN M. LEONARD, AHMED A. HUSSEIN, NAMRITA LALL. 2009. Bioactivities of *Plectranthus ecklonii* constituents. Natural Product Communications 4, 1177-1180.

M.A. NYILA, C. M. LEONARD, A. A. HUSSEIN, N. LALL. Bioactivity of antilisterial compounds isolated from *Acacia karroo* (Manuscript submitted).

APPENDIX 3- Additional information

Questionnaire for the traditional healers

1. How long you have been practising traditional healing?

2. Which medicinal plants do you use to treat, when you have patients complaining for fever, diarrhoea, severe headaches, pulsating fever, nausea, vomiting, stiffness of the neck and back, etc.?

3. How do you determine which medicinal plant should be used?

4. From where do you collect the appropriate medicinal for your patient and where do you collect these medicinal plants?

5. Which equipment do you use to harvest the plant parts (e.g. leaves, stem or roots) before you prepare the medicine for your patients?

6. Do you request your fellow traditional healers to give you the medicinal plants which you are unable to get from your usual place of collection?

7. How do you determine the dosage to be used?

8. Do you disclose the name of the medicinal plant that you have prepared, to your patient?

9. How do you prepare medicine?

10. If your patient's condition does not improve what do you do? Do you refer your patients to another traditional healer or do you change the medicinal plant or the dosage?

11. If you see that the conditions of your patients deteriorate do you refer them to the Western medical practitioners or the hospital?

12. How many patients visit you, on average, daily or monthly?

13. Where do most of your patients come from?

14. How do patients from far know about your practise?

16. Do you get a feedback from your patients after a successful treatment?