

## Chapter 3

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

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

### 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  $\beta$ -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  $\beta$ -sitosterol and epigallocatechin was reduced from 25 $\mu$ m as observed in untreated cells to < 10  $\mu$ m 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

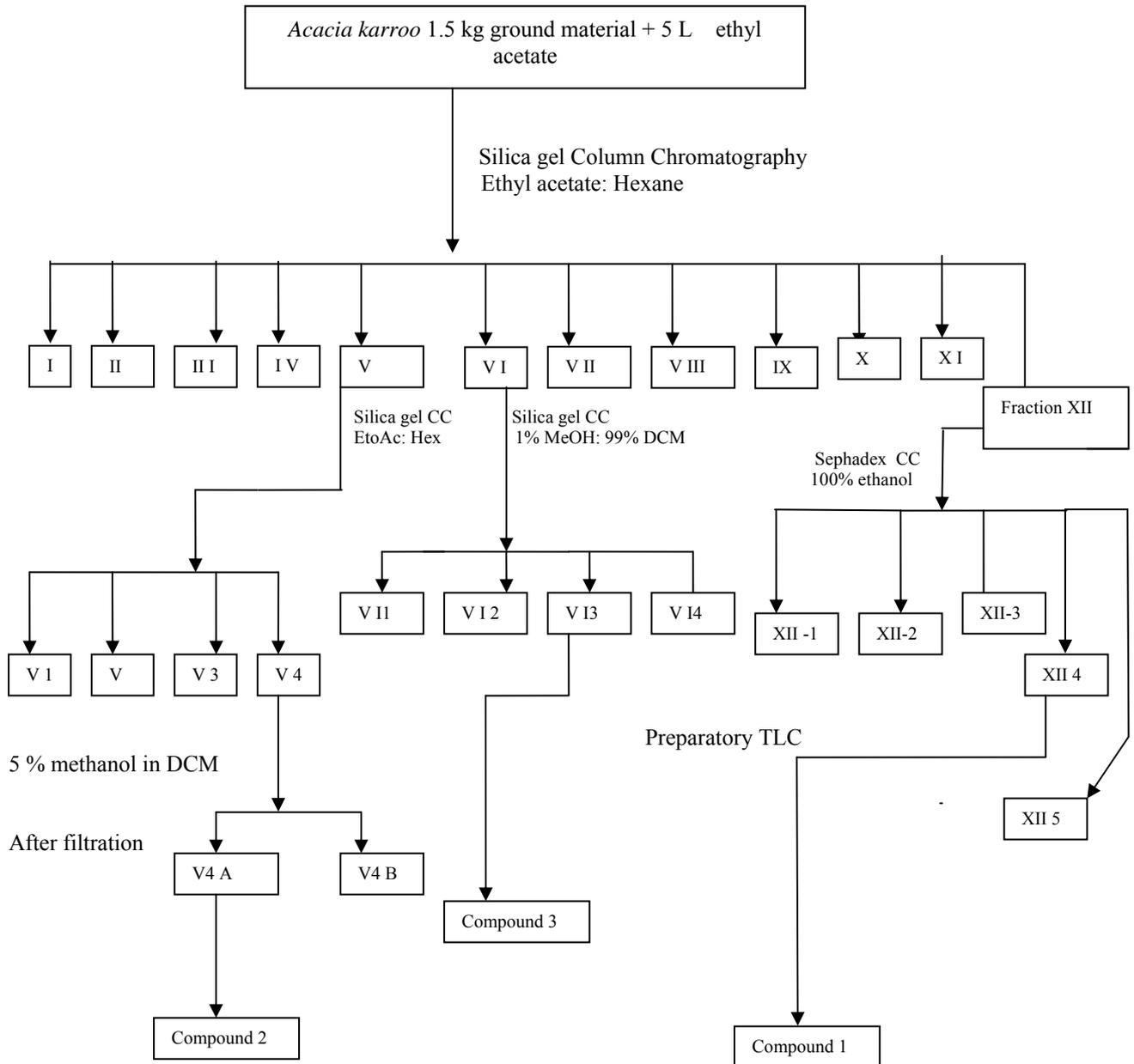
was performed to obtain a concentration range of 25- 0.01 mg/ml for the extracts. For compounds the antibiotic erythromycin (Merck) at concentrations ranging from 150 - 0.29 µg/ml served as a positive drug control. Hundred microlitres of bacterial inoculum  $10^6$  CFU/ml of *L. monocytogenes* was added 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 ( $\pm 25$  °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).

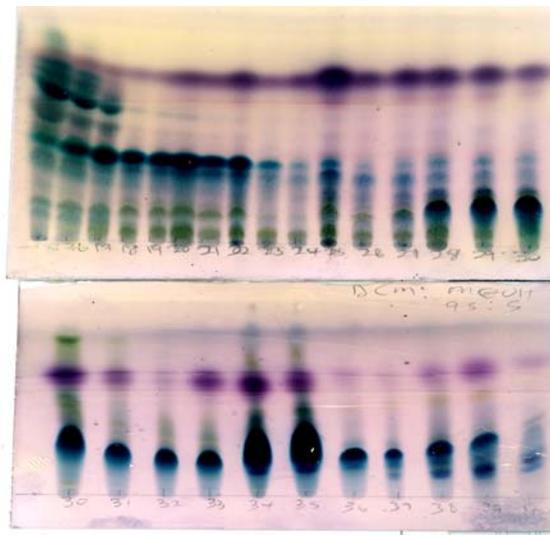


**Figure 3.1** Schematic representation of the isolation process of the bioactive compounds from *Acacia karroo*

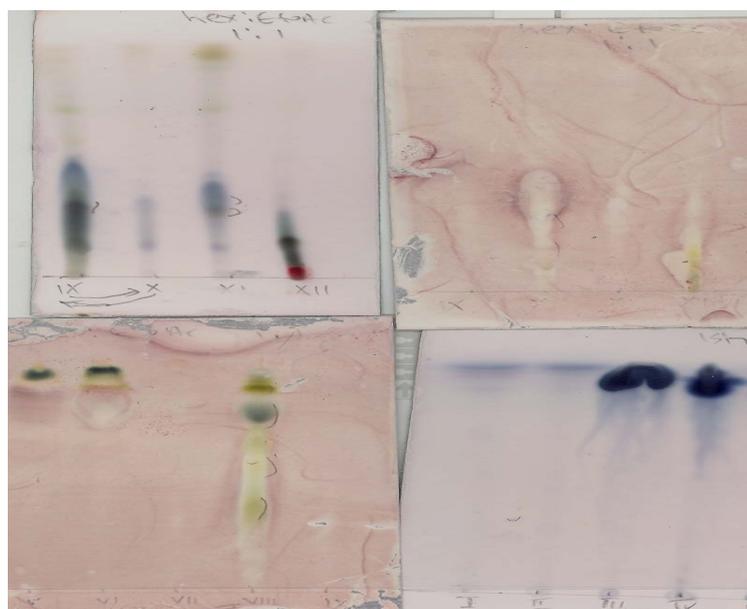
Direct bioassay on the TLC plate was done by applying 20  $\mu$ l of the plant extract (50mg/ml) to the silica gel 60 F<sub>254</sub> plate (Simonovska & Vovk, 2000). The plates were developed using ethyl acetate: ethanol (9:1, v/v) eluent and then dried carefully. The 24-hour *L. monocytogenes* in TSB 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 %). <sup>1</sup>H NMR and <sup>13</sup>C NMR spectra were recorded using a Bruker ARX 300 or a Bruker Avance DRX 500 MHz using CDCl<sub>3</sub>, and DMSO-*d*<sub>6</sub> 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  $\mu\text{g/ml}$ . for pure compounds ranged from 1.5 200  $\mu\text{g/ml}$  . The positive drug ‘Zearalenone’, at a final concentration of 1.25  $\mu\text{g/ml}$ , 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% ( $\text{IC}_{50}$ ) 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 spectrophotometrically 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  $\text{IC}_{50}$  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  $\text{IC}_{50}$  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. monocytogenes* 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<sub>2</sub>SO<sub>4</sub> reagent on TLC was

eluted from Fraction XII. The compound was identified on the basis of  $^1\text{H-NMR}$  data (Figure 3.3). The  $^1\text{H-NMR}$  data of **1** exhibited signals identical with that of epicatechin which exhibited signals of 6 protons, at  $\delta$  4.85 assigned to H-2, a proton signal  $\delta$  4.18 ppm, to H-3, and the two protons signals at 2.74 and 2.80 ppm were assigned to protons 4 $\alpha$  and 4 $\beta$  respectively, signal at  $\delta$  6.01 was assigned to H-6 and the signal at 5.90 to H-8 (Okushio *et al.*, 1998). Aromatic signals at  $\delta_{\text{H}}$  7.02, 6.75, and  $\delta_{\text{H}}$  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  $\delta_{\text{H}}$  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).

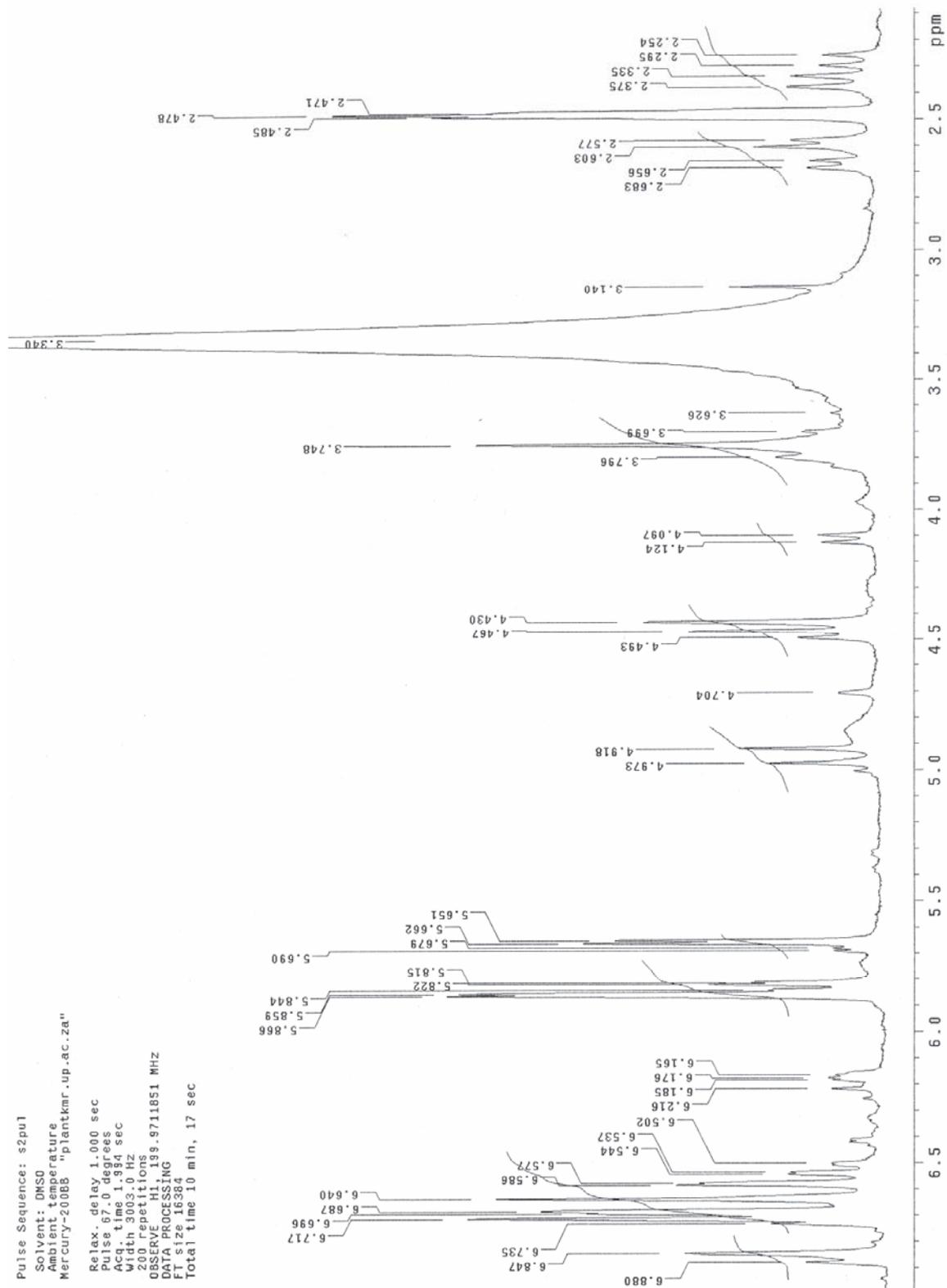


Figure 3.3  $^1\text{H}$ NMR spectrum of compound 1 (epicatechin) isolated from *A. karroo*.

### **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 <sup>1</sup>H-NMR data (Figure 3.4). <sup>1</sup>HNMR spectra showed peaks at δ<sub>H</sub> 5.35 corresponding to H-6, δ<sub>H</sub> 3.79 to h-3 in addition to six methyl group signals at δ<sub>H</sub> 1.00, 0.67 (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 <sup>13</sup>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*.

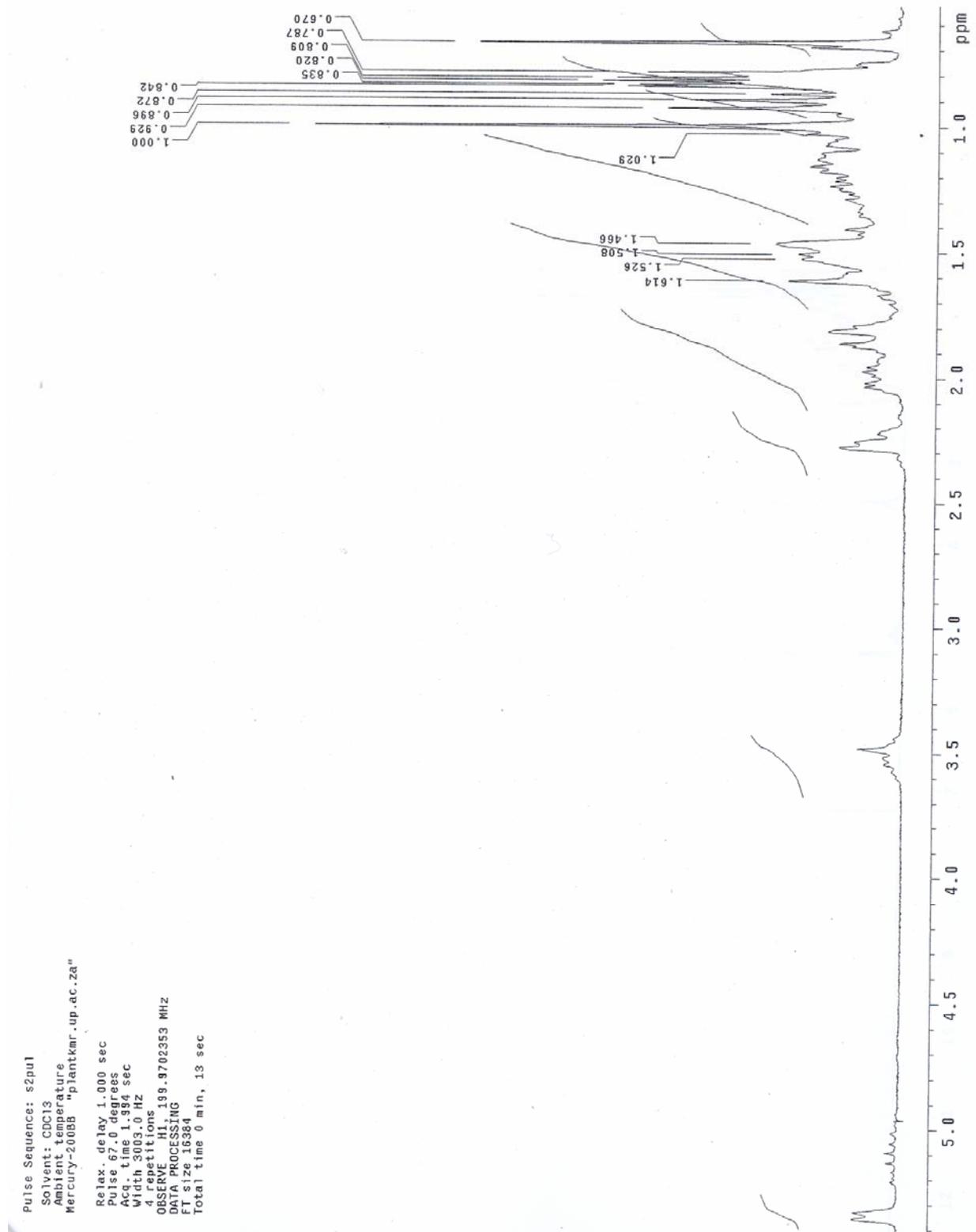


Figure 3.4 <sup>1</sup>H NMR spectrum of compound 2 ( $\beta$ -sitosterol) isolated from *A. karroo*.

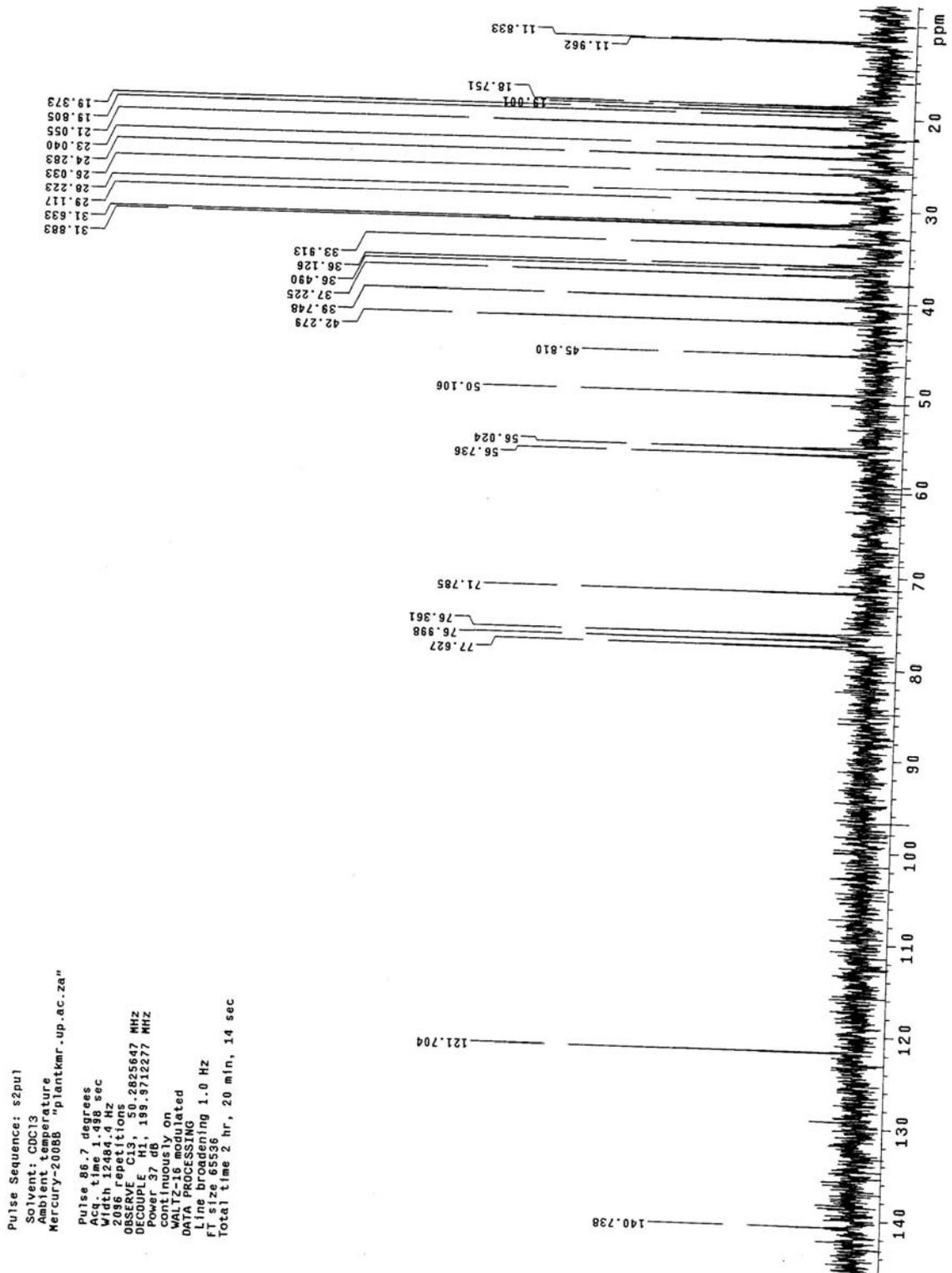


Figure 3.5 <sup>13</sup>C NMR spectrum of compound 2 (β-sitosterol) isolated from *A. karroo*.

### 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 <sup>1</sup>H-NMR spectrum (Figure 3.6) which showed four aromatic protons at  $\delta_{\text{H}}$  5.68 (d, J = 2.3), 5.87 (d, J = 2.3), 6.38 (2H, d, J= 0.6), two methyl protons at  $\delta_{\text{H}}$  4.86 (d, J= 0.8 and 3.77 (s br), methylene protons at  $\delta_{\text{H}}$  2.45 (J = 4.4) and 2.33 (J= 3.2). The <sup>13</sup>C-NMR spectra (Figure 3.7) indicated the presence of two methyl carbons attached to an oxygen function ( $\delta_{\text{C}}$  78.6, 65.6), a methylene carbon ( $\delta_{\text{C}}$  28.8 t), 12 aromatic carbons  $\delta_{\text{C}}$  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  $\delta_{\text{H}}$  4.86 and 3.77 is 2.3=Hz which indicated  $\beta$  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 *Elaeagnus 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.

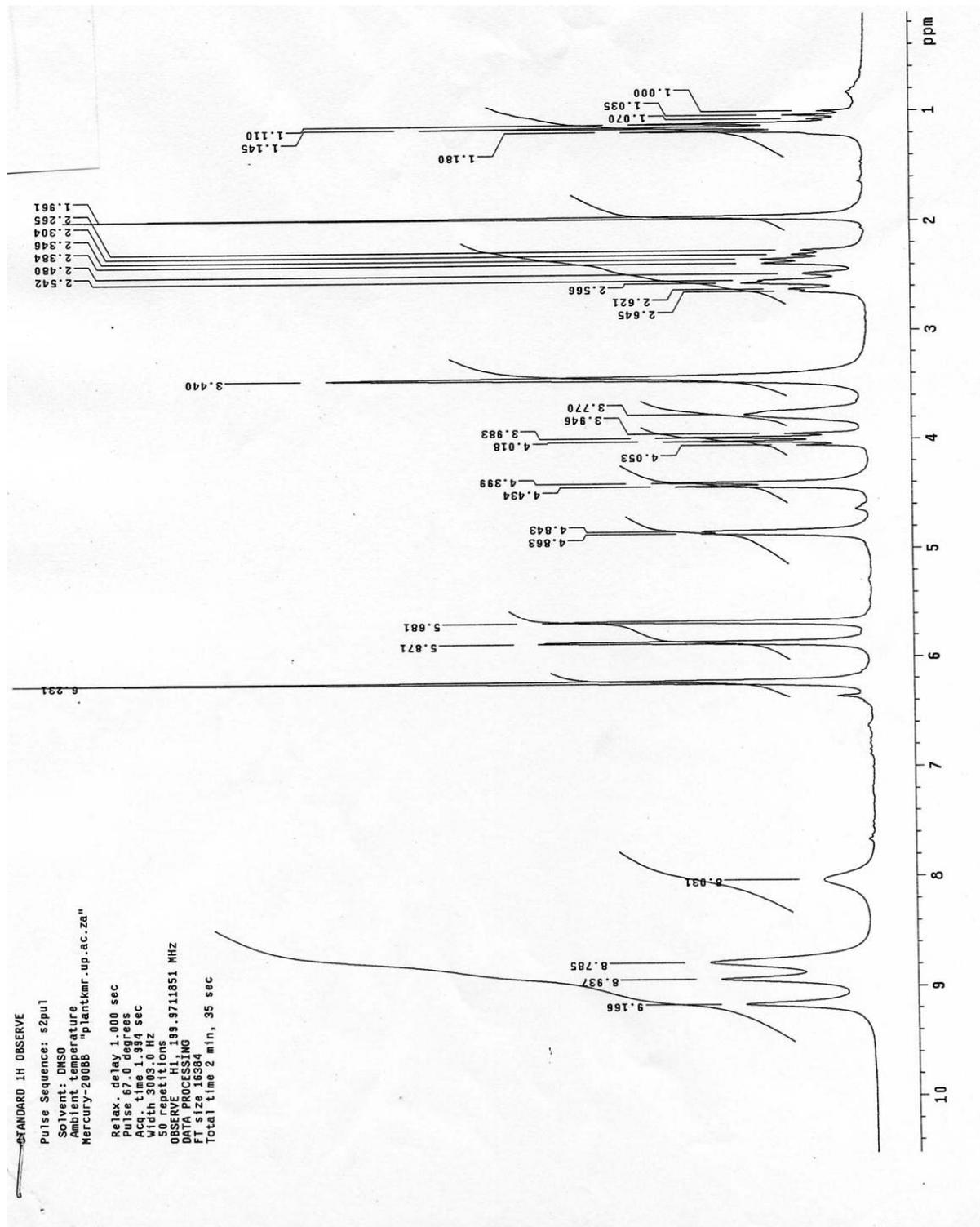


Figure 3.6 <sup>1</sup>H NMR spectrum of compound **3** (epigallocatechin) isolated from *A. karroo*.

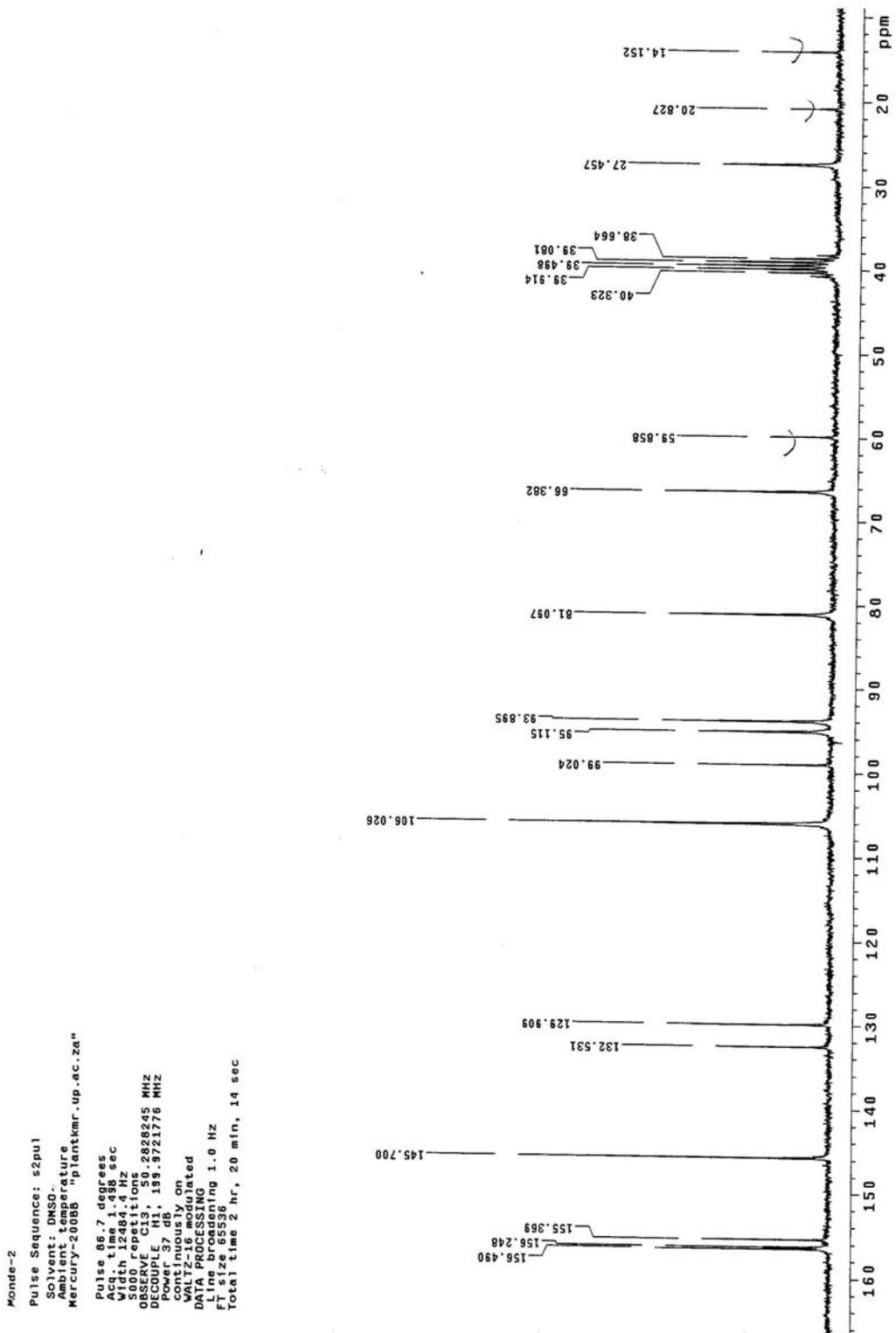
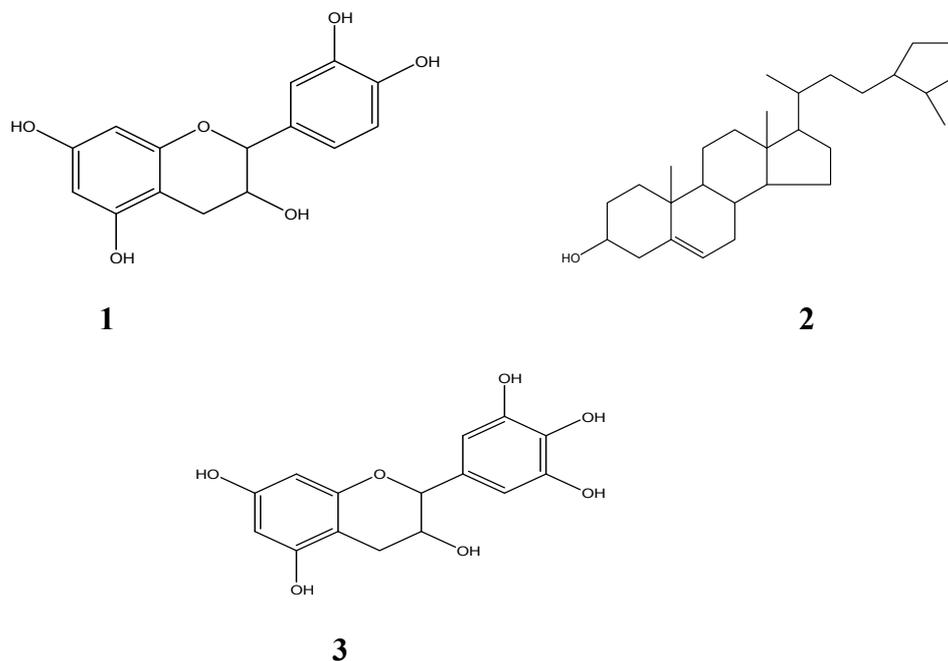


Figure 3.7  $^{13}\text{C}$ NMR spectrum of compound 3 (epigallocatechin) isolated from *A. karroo*.



**Figure 3.8** Chemical structures of compounds isolated from the ethyl acetate extract of the leaves of *A. karroo*, epicatechin(1) ,  $\beta$ -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 absinthium* 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 <sup>a</sup>	Zone of inhibition (mm) Ethyl acetate	Zone of inhibition (mm) Chloroform	Voucher specimens
<i>Aloe arborescens</i>	L	9	8	MN 5
<i>Acacia karroo</i>	L	14	12	MN 15
<i>Artemisia afra</i>	L	NZ <sup>b</sup>	NZ	MN 7
<i>Clivia miniata</i>	W	NZ	NZ	MN 3
<i>Datura stramonium</i>	L	NZ	NZ	MN 8
<i>Drimia altissima</i>	R	NZ	NZ	MN 14
	L	NZ	NZ	
<i>Eucomis autumnalis</i>	B	12	13	MN 11
<i>Gomphocarpus fruticosus</i>	L	NZ	NZ	MN 1
<i>Heteromorpha arborescens</i>	S	NZ	NZ	MN 4
	L	NZ	NZ	
<i>Plectranthus ecklonii</i>	L	15	12	PRU 96396
<i>Senecio inornatus</i>	L	8	8	MN 9
	S	8	NZ	
<i>Tulbaghia violacea</i>	L	NZ	NZ	MN 12
<i>Ziziphus mucronata</i>	L	NZ	NZ	MN 10
Erythromycin (150 µg/ml) (drug control)			14 mm	

<sup>a</sup>plant part used: (W) whole plant; (L) leaves; (R) roots; (B) bulb; (S) stem

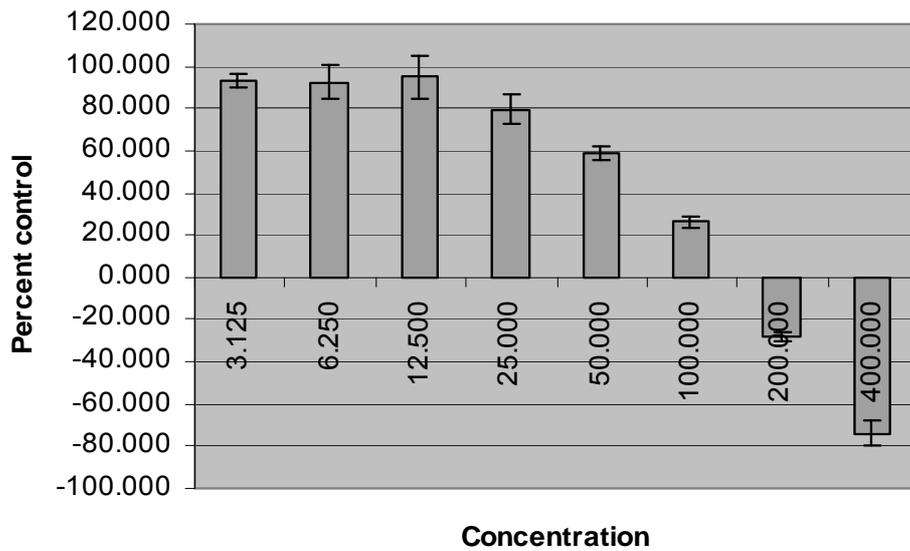
<sup>b</sup>NZ : No zone of inhibition observed (extract not active against *L. monocytogenes*)

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 antibacterial activity against *Proteus vulgaris* and *S. Aureus* (Mori *et al.*, 1987). The flavonoid, pinocembrin has been previously reported to have antilisterial activity (Välilmaa *et al.*, 2007). Other flavonoids that have been reported to exhibit both bactericidal and bacteriostatic activity against several strains of *L. monocytogenes* are hydroxycinnamic 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<sub>50</sub> value of >200 µg/ml. β-sitosterol killed less than 50% cells at highest concentration the IC<sub>50</sub> 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<sub>50</sub>) value as compared to the control drug (Figures 3.9 and 3.10).

The selectivity index (SI = IC<sub>50</sub>/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.  $\beta$ -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.

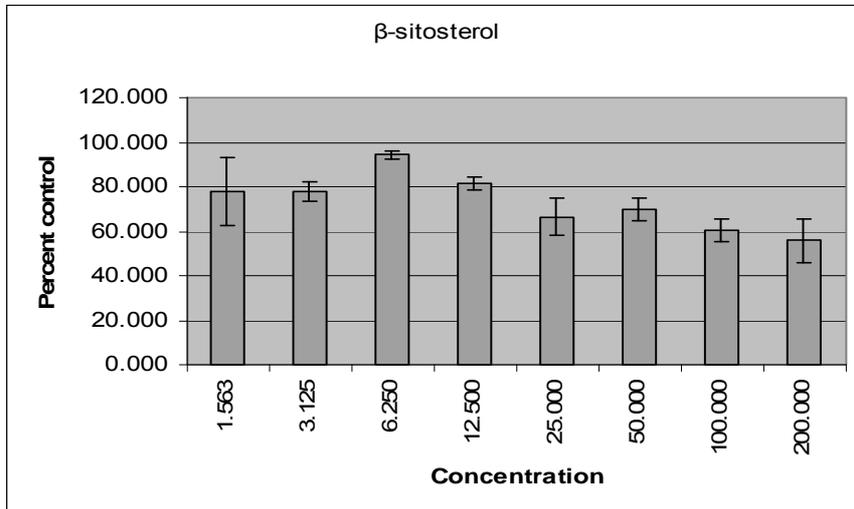
**Table 3.2** The minimum inhibitory concentration and the minimum bactericidal concentration of the crude extracts of *A. karoo*, *E. autumnalis*, *S. inornatus*, *A. arborescens* and *P. ecklonii* against *L. monocytogenes* and their fifty percent inhibitory concentration against Vero monkey cell lines.

Compounds/extracts	MIC (mg/ml)	MBC (mg/ml)	*IC <sub>50</sub> (µg/ml) ± SD	SI value = IC <sub>50</sub> / 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
<i>A. karoo</i> (ethyl acetate) (chloroform)	3.1	3.1	45.49 ± 7.86	0.015
	6.25	6.25	NT <sup>a</sup>	NA
<i>P. ecklonii</i> (ethyl acetate) (chloroform)	0.5	1.0	30.125	0.06
	6.25	6.25	NT	NA
<i>E. autumnalis</i> (ethyl acetate) chloroform	12.5	12.5	NT	NA
	12.5	12.5	NT	NA
<i>S. inornatus</i> (ethyl acetate) (chloroform)	12.5	12.5	108.4 ± 0.995	0.009
	12.5	12.5	99.94 ± 4.191	0.008
<i>D. altissima</i> (ethyl acetate) (chloroform)	12.5	12.5	NT	NA
	12.5	12.5	NT	NA
<i>A. arborescens</i> (ethyl acetate) (chloroform)	12.5	12.5	>400.0	0.032
	12.5	12.5		NA
Erythromycin (antibacterial drug control)	1.7 x 10 <sup>-3</sup>	1.7 x 10 <sup>-3</sup>	14.38	8.46
Zearalenone (positive drug for cytotoxicity)	NA	NA	2.318 ± 0.301	NA

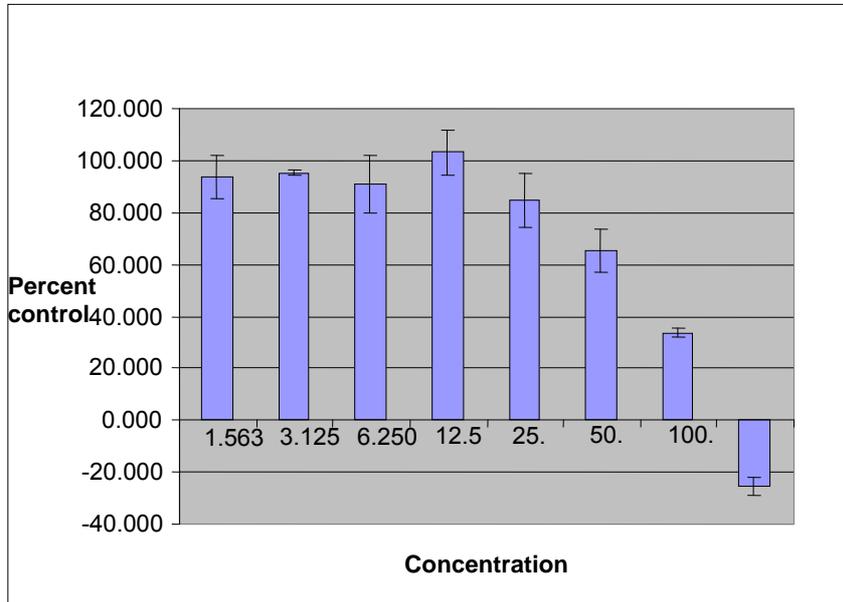
\* IC<sub>50</sub>: Fifty percent cytotoxic values ; <sup>a</sup> NT : Not tested; NA : Not applicable

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)).



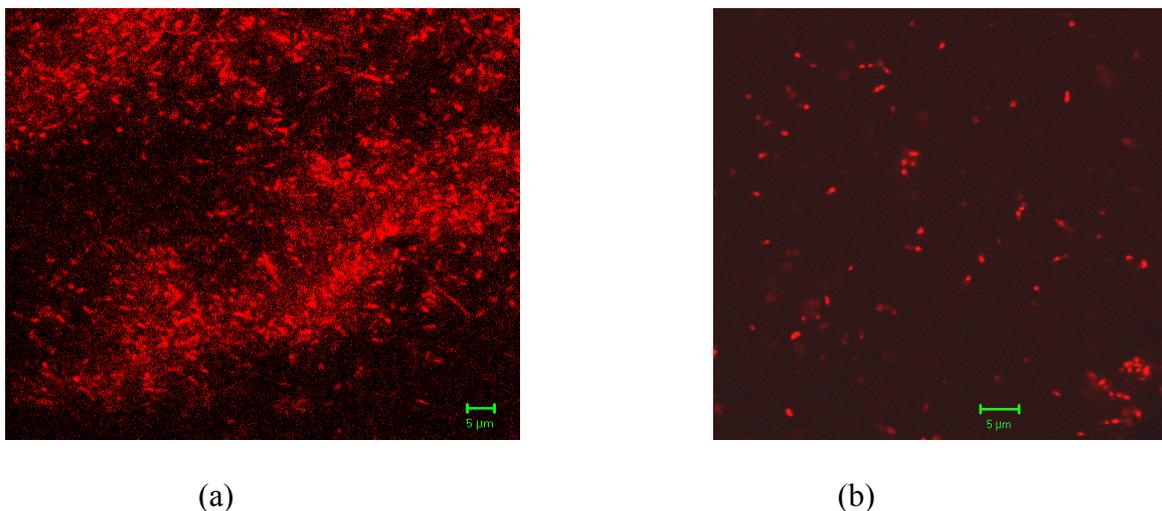
(a)



(b)

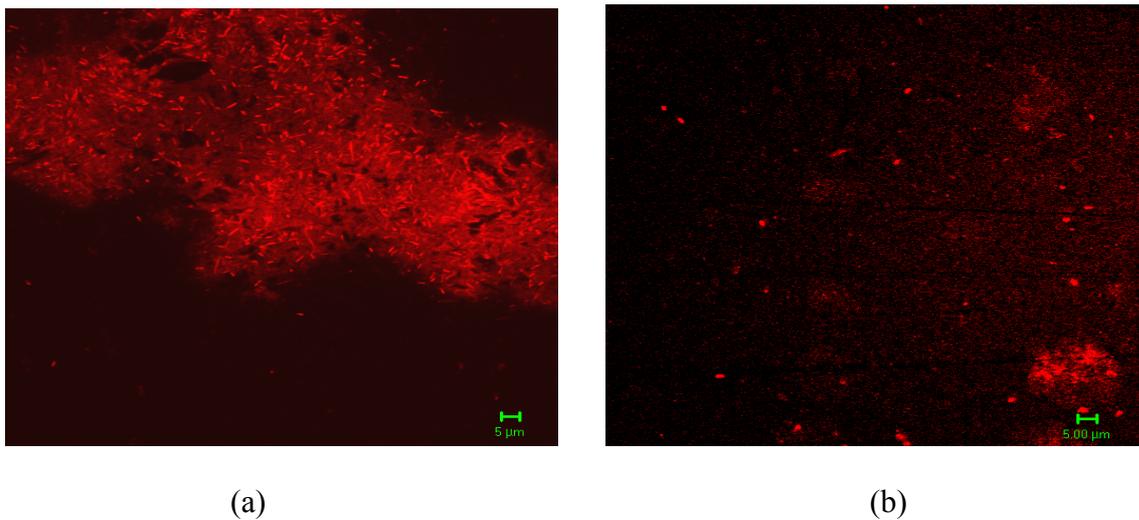
**Figure 3.10** Dose response curve of (a)  $\beta$ -sitosterol (**2**), (b) epigallocatechin (**3**) compounds against Vero cell lines.

The crude extract showed a slight disruption of listerial biofilm (Figure 3.12 (a)). The average thickness of the treated biofilms was 1  $\mu\text{m}$  and the maximum thickness was 3  $\mu\text{m}$ . These results are in agreement with the previous report who reported that the use of benzalkonium chloride, a quaternary ammonium compound reduced the formation of *L. monocytogenes* biofilms (Romanova *et al.*, 2007). In the present study,  $\beta$ -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  $\mu\text{m}$  in untreated cells to < 10  $\mu\text{m}$  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*.



**Figure 3.11.** CSLM images of *L. monocytogenes* (LMG21263) biofilms without treatment (a) and after treatment with  $\beta$ -sitosterol (b). *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  $\mu\text{m}$ ).

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  $\beta$ -sitosterol and epigallocatechin was reduced from 25  $\mu\text{m}$  as observed in untreated cells to  $< 10 \mu\text{m}$  in diameter in the treated cell.



**Figure 3.12** CSLM images of *L. monocytogenes* (LMG21263) biofilms after treatment with *A. karroo* (a) and after treatment with epigallocatechin (b). *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  $\beta$ -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. monocytogenes* 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; Kolaczowski *et al.*, 2009).

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