

## Chapter 5

# Determination of antimicrobial, cytotoxic and antioxidant activities of extracts

### 5.1 Introduction

Effective therapies for HIV infection are being sought far and wide, in the natural world as well as in laboratories. *In vivo* anti-HIV studies of glycyrrhizin, from glycyrrhiza plants (the source of licorice), extended the life of retrovirus-infected mice from 14 to 17 weeks (Watanbe *et al.*, 1996). A crude extract of the cactus *Opuntia streptacantha* had a marked antiviral effect *in vitro*, and toxicity studies performed in mice, horses, and humans found the extract to be safe (Ahmad *et al.*, 1996).

Human mycoses, especially in immunocompromised patients, are not always successfully treated due to the ineffectiveness or toxicity of the available antifungal drugs (Barrett-Bee and Ryder, 1992). Similarly some bacterial infections, especially those produced by *Staphylococcus aureus*, may be difficult to manage. Therapy with several types of antibiotics (Hiramatsu *et al.*, 1997) is frequently accompanied by side effects and microbial resistance, such as is the case with methicillin-resistant *Staphylococcus aureus* (MRSA). New and potent antimicrobial agents are still needed and should be actively sought (Chambers, 1997; Selitrennikoff, 1992). Belachew (1993) showed that the direct aqueous extract of the leaf of *C. paniculatum* showed bacterial inhibition against *S. aureus* which was better than that shown by the antibiotic neomycin.

Cytotoxicity measures the toxicity of extracts to cells. It is measured as functions of fundamental biochemical pathways leading to cell death (Mosmann, 1983). Only one in ten drug candidates make it through the development process. One-third of these failures are due to unacceptable toxicity levels (Ricerca, 1998). A cytotoxicity assay is a rapid and cost-effective tool to sort out the likely failures before a compound is entered into the costly development process and to help choose the optimal candidate (Wallin and Arscott, 1998). Cytotoxicity testing is a rapid, standardized, sensitive and inexpensive means to determine whether a material contains significant quantities of biologically harmful extractables. The high sensitivity of the tests is due to the isolation of the test cells in cultures and the absence of the protective mechanisms that assist cells within the body (Ricerca, 1998).

## 5.2 Methods

The leaf, stem bark and root bark extracts were tested for various biological activities, and initial concentrations for each extract were 100 mg/ml.

### 5.2.1 Antiviral activity

The procedure for this test described in section 3.9.1 was followed to determine antiviral activity of the extracts. The virucidal activity of the diluted extract was calculated by comparing the infectivity titre obtained in the absence of the extract ( $\log_{10}$  TCID<sub>50</sub> of the controls) while the infectivity titre was determined after the interaction of virus and extract. The difference between the two values was equal to the reduction of the infectivity titre. The Karber method was used for calculating the TCID<sub>50</sub> (Karber, 1931).

### 5.2.2 Antibacterial activity

The procedure for this test is described in section 3.10.1.

### 5.2.3 Bioautography

The method used for this test is described in section 3.10.3.

### 5.2.4 Antioxidant activity

The TLC DPPH spray method described in section 3.13 was used to determine the presence of antioxidant compounds in the extract.

### 5.2.5 Cytotoxicity (MTT assay)

After scoring the percentage cell damage in the antiviral test, with the use of a light microscope, the MTT method was used as an additional test to determine cytotoxicity. This procedure is described in section 3.9.1.4.

## 5.3 Results

### 5.3.1 Antiviral and cytotoxic activities of extracts

Fig 5.1 shows the antiviral and cytotoxic activities of the acetone extract of the leaves. The virus used was feline herpesvirus (type 1) and the host cells were Crandell feline kidney cells (CRFK). The cells were destroyed by virus (plate C) up to the fifth column of the microtitre plate (i.e. up to a dilution of  $10^{-5}$ ). The extract alone (plate B) damaged cells in the first column only (at a dilution of  $10^{-1}$ ). For the extract antiviral test (A), the cells were damaged in the first column and partly in the second column. It was concluded that the extract diminished cell damage by the virus by three 10-fold dilutions (a thousand-fold). The deep purple colour indicates wells where the cells are viable and the lighter coloured wells are those in which the cells were partly destroyed.

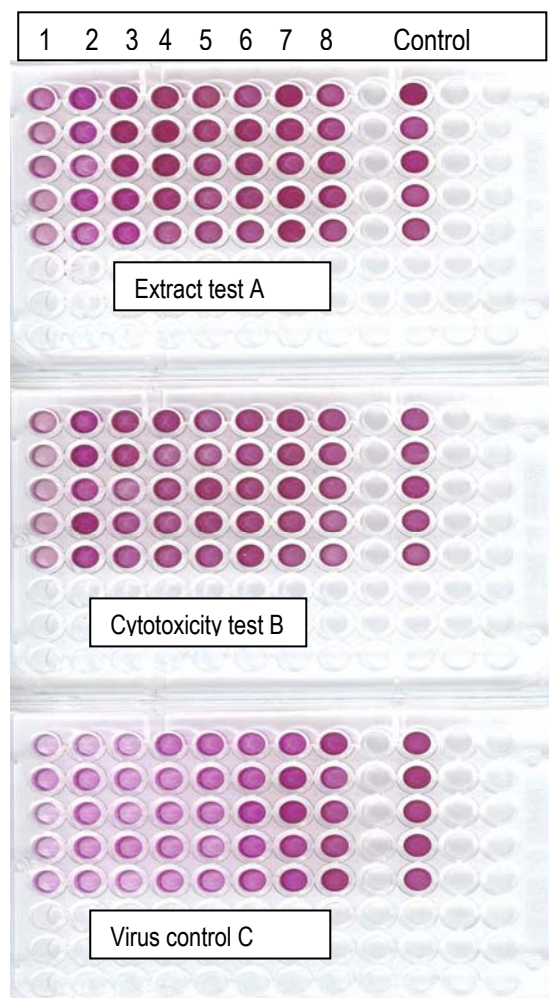


Fig 5.1. Microplate representation of acetone leaf extract test (A), cytotoxicity (B) and virus control (C)

The results of the various extracts tested are shown in Table 5.1.

Table 5.1. Antiviral activity of *C. paniculatum* extracts against FHV-1

Part of plant	Extract test (b)	Virus titre (a) (TCID <sub>50</sub> )	Antiviral activity (c = a - b)
Leaf (acetone)	10 <sup>-1.5</sup>	10 <sup>-4.5</sup>	10 <sup>3</sup>
Leaf (water)	10 <sup>-2.5</sup>	10 <sup>-5.5</sup>	10 <sup>3</sup>
Leaf (80% methanolic)	10 <sup>-1.5</sup>	10 <sup>-4.5</sup>	10 <sup>3</sup>
Stem bark (water)	10 <sup>-2.5</sup>	10 <sup>-6.1</sup>	10 <sup>3.6</sup>
Root bark (water)	10 <sup>-2.5</sup>	10 <sup>-5.9</sup>	10 <sup>3.4</sup>

The results are reported as tissue culture infectious dose 50% (TCID<sub>50</sub>) in log<sub>10</sub> values. The criterion of efficacy for virucides stipulated by both the DVV (1984) and AFNOR (1989) is a 4.0 log<sub>10</sub> reduction in virus titre. However, Sattar and Springthorpe (1991) suggested that a 3.0 log<sub>10</sub> reduction, if achieved under realistic conditions, is an adequate measurement of product efficacy.

### 5.3.2 Antibacterial activity

Leaves of the plant were extracted in 8 solvents and their MICs determined against four bacterial organisms. The lowest average MIC value was obtained for DCM and THF (0.275 mg/ml) extracts (Table 5.2). The highest MIC values were those of the water and hexane extracts. This is similar to the situation in practically all members of the Combretaceae examined to date. *E. faecalis* was the most sensitive bacterium while the least sensitive species to the extracts was *S. aureus*. The MIC values for the different extractants ranged from 0.156 to 2.5 mg/ml. There was only one value of 2.5 mg/ml and this was for the water extract against *S. aureus*. All the extracts had a substantial antibacterial activity towards the four bacteria examined with average values of 0.47, 0.59, 0.57 and 0.29 mg/ml for *E. coli*, *S. aureus*, *P. aeruginosa* and *E. faecalis*. Plant extracts are frequently much more active against Gram-positive bacteria (Vlietinck *et al.*, 1995), but this was not true for *C. paniculatum* extracts.

The highest total activity according to Kotze and Eloff (2002) while investigating *C. microphyllum* was for the methanol extract followed by the DCM extract, while for *C. paniculatum*, the highest total activity was for the water extract followed by the methanol extract. The total activity values for *C. paniculatum* leaf extracts are reported in Table 5.2.

Table 5.2. MIC (mg/ml) and total activity (TA, ml/g) values of *C. paniculatum* leaf extracts prepared using eight extractants

	Acetone		CCl <sub>4</sub> *		EtOH		C <sub>6</sub> H <sub>12</sub>		MeOH		DCM		THF		water	
	MIC	TA														
Qty (mg) extracted per gram	29		27		43		23		194		15		47		246	
<i>E. coli</i>	0.315	92	0.63	43	0.315	136	1.25	18	0.315	615	0.315	47	0.315	149	0.315	780
<i>S. aureus</i>	0.63	46	0.63	43	0.63	68	1.25	18	0.63	310	0.315	47	0.315	149	0.315	780
<i>P. aeruginosa</i>	0.156	185	0.315	85	0.156	275	0.63	36	0.315	615	0.315	47	0.156	301	2.5	98
<i>E. faecalis</i>	0.156	185	0.156	175	0.315	136	0.315	73	0.63	310	0.156	96	0.315	149	0.315	780
Average	0.313	127	0.432	86.5	0.354	153.7	0.861	36.3	0.47	462.5	0.275	59.3	0.275	187	0.861	609.5

\* CCl<sub>4</sub> = carbon tetrachloride, EtOH = ethanol, C<sub>6</sub>H<sub>12</sub> = hexane, MeOH = methanol, DCM = dichloromethane, THF = tetrahydrofuran

### 5.3.3 Bioautography of extracts

Figure 5.2 shows a bioautographic plate of extracts prepared using 10 solvents, developed in CEF.

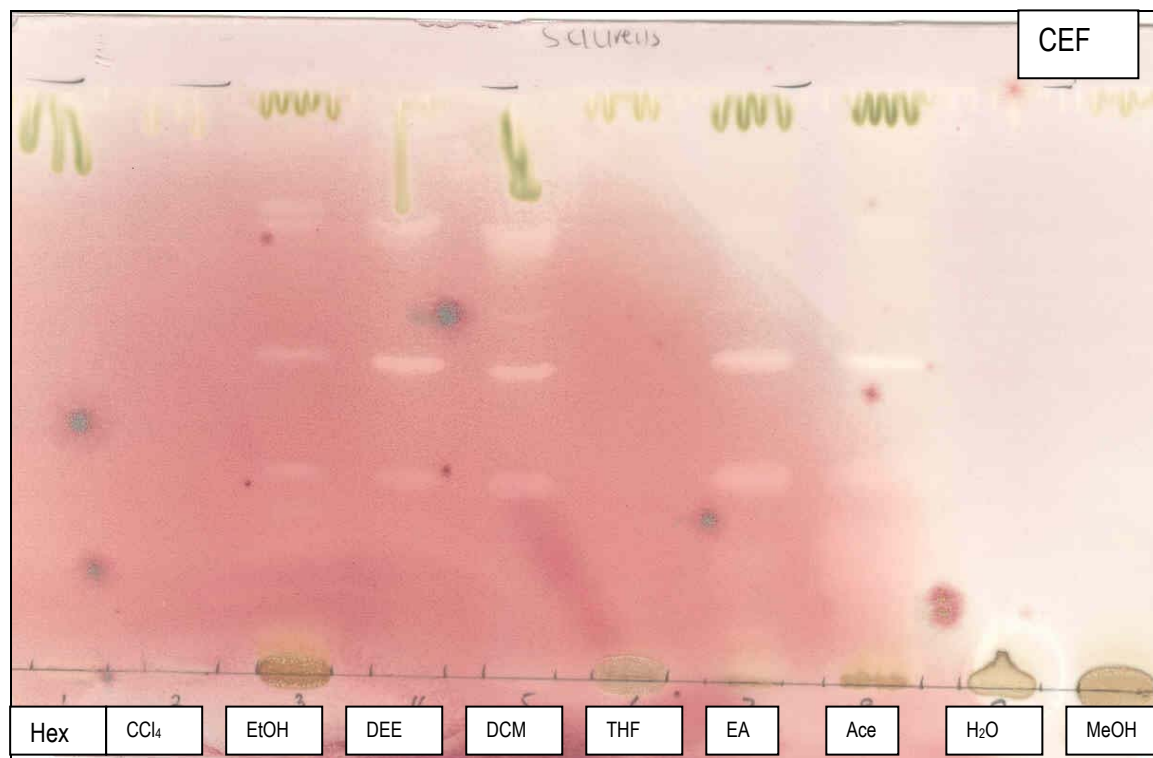


Fig 5.2. Bioautography of ten different leaf extracts of *C. paniculatum* against *S. aureus* in CEF

The solvents from left to right are: hexane, carbon tetrachloride, ethanol, diethyl ether, dichloromethane, tetrahydrofuran, ethyl acetate, acetone, water and methanol. Inhibition zones are the clear zones on the purple background.

### 5.3.4 Antioxidant activity of extracts

Results of the antioxidant activity of extracts of leaves prepared using five different solvents are shown in Fig 5.3, and those of the root bark extracts in Fig 5.4. The clear zones in the purple background are areas where components exhibit antioxidant activity after spraying with DPPH. This is a rapid, qualitative method of determining antioxidant activity of extracts.

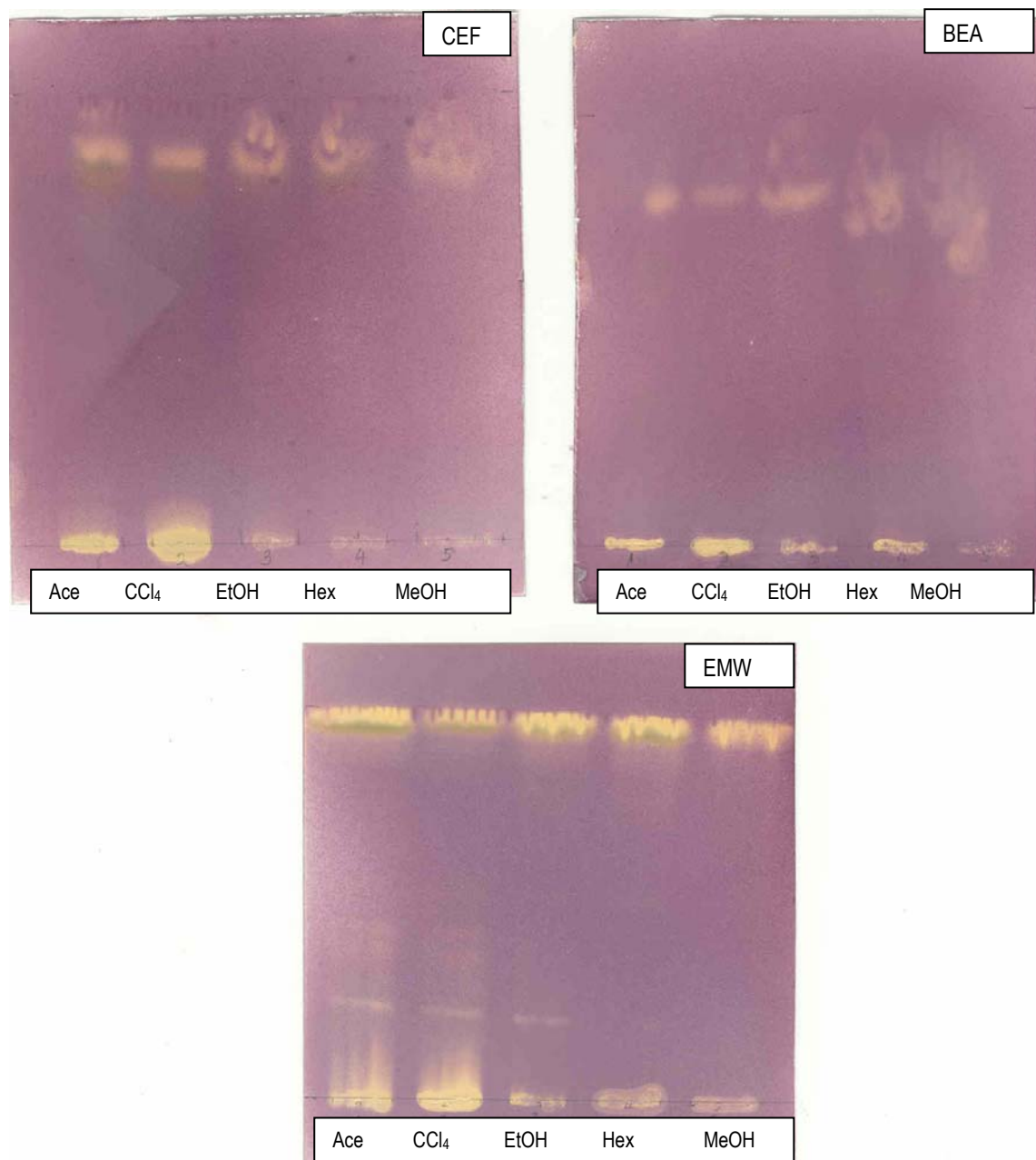


Fig 5.3. DPPH spray of leaf extracts in different solvents

The chromatograms were developed in three solvent system (CEF, BEA and EMW) and sprayed with DPPH.

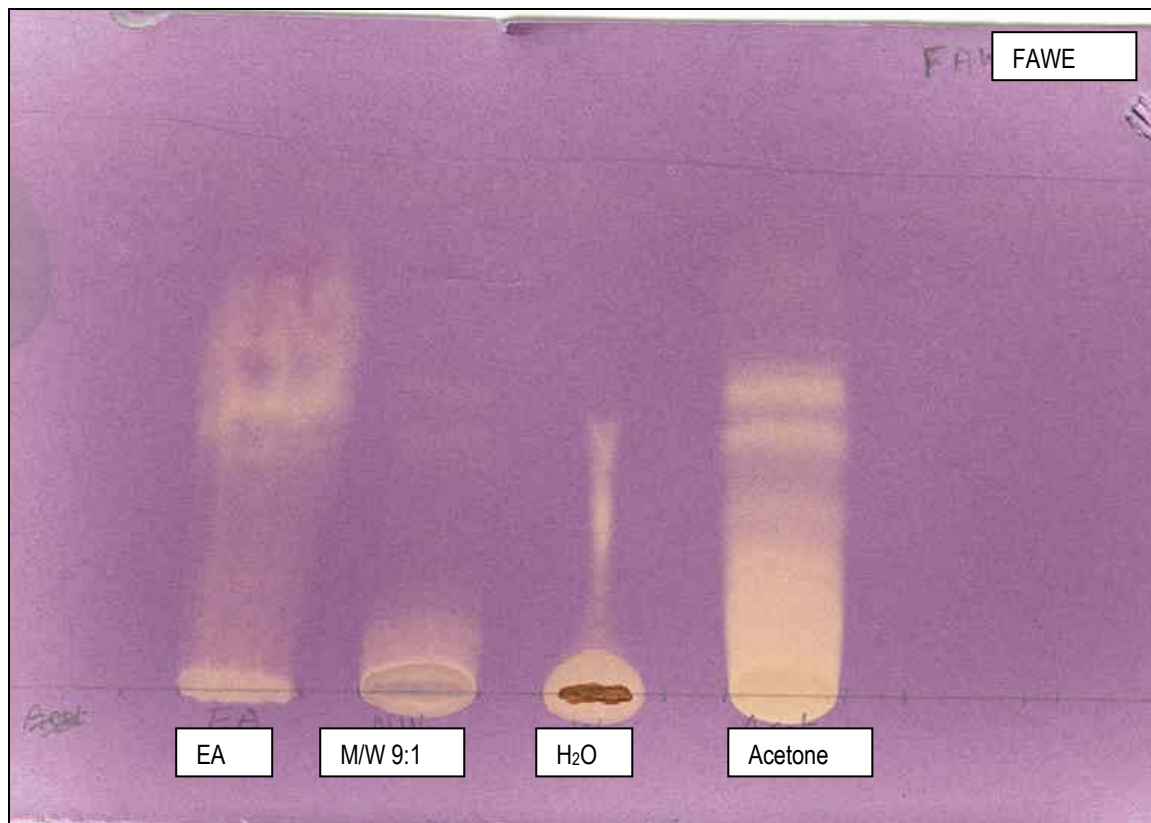


Fig 5.4. DPPH spray of the root bark extracts of *Combretum paniculatum* prepared using four solvents

## 5.4 Discussion

The antiviral activity of the leaf, stem bark and root bark was determined microscopically by examination for cytopathic effect (CPE) and also with the aid of the colorimetric MTT assay. The antiviral activity was the difference between the virus titre in the absence and in the presence of the extract ( $\delta \log_{10}$  TCID<sub>50</sub>/ml). CPE was determined by microscopic examination of the cells and the results used to calculate the TCID<sub>50</sub> values.

The antiviral activity of the stem bark was  $10^{3.6}$  which represented a reduction in viral titre of  $3.6 \log_{10}$ . This was followed by the root bark with a TCID<sub>50</sub> of  $10^{3.4}$  representing a  $3.4 \log_{10}$  reduction. The criterion of efficacy for virucides stipulated by both the DVV (1984) and AFNOR (1989) is a  $4.0 \log_{10}$  reduction in virus titre. The effective concentration 50 (EC<sub>50</sub>) for the acetone extract of the leaf was  $2.8 \mu\text{g/ml}$  against feline herpesvirus (FHV-1). The EC<sub>50</sub> was determined by calculating the concentration where 50% of the cells were destroyed. This was done by visual observation with a light microscope.



It is very interesting that water and acetone extracts had a similar antiviral activity. This is contrary to our experience with antibacterial and antifungal activity of Combretaceae extracts, where aqueous extracts were always inactive. The antiviral activity found in the extracts prepared using polar extractants indicates that tannins or polysaccharides may be responsible for the antiviral activity.

Asres *et al.* (2001) reported that the acetone extract of the leaf of *C. paniculatum* inhibited the replication of HIV-2 with an EC<sub>50</sub> value of 3.0 µg/ml and selectivity index of 32. This confirms that the acetone extract of the leaves of *C. paniculatum* inhibits the replication of both HIV-2 (Asres *et al.*, 2001) and FHV-1 (shown in the present study). Due to the good activity for the extracts, it is expected that the pure compounds, once inactive compounds have been removed, will be more active.

The antibacterial MIC values obtained for *C. paniculatum* were lower than those obtained for other members of the Combretaceae family. The average MIC values ranged between 0.28 and 0.86 mg/ml. Eloff (1999) found that members of the Combretaceae inhibited bacterial growth and MIC values ranged from 0.1 to 6 mg/ml with an average of 2.01 mg/ml. Gram-positive strains were slightly more sensitive with an average MIC value of 1.8 mg/ml while activity of Gram-negative strains averaged 2.22 mg/ml.

In a publication investigating 63 medicinal plant species from Ethiopia using the agar plate well-diffusion method using a sample concentration of 1000 µg/ml, Belachew (1993) showed that the direct aqueous extract of the leaves of *C. paniculatum* had activity against *S. aureus* superior to that obtained with the antibiotics neomycin and nystatin. Bioautography of the crude leaf extracts showed compounds with bacterial growth inhibition for acetone and ethyl acetate extracts in this study.

The leaf extracts were spotted on TLC, developed and sprayed with 0.2% DPPH to reveal antioxidant activity. Almost all the fractions had antioxidant compounds, but acetone and carbon tetrachloride had bigger zones, indicating a higher antioxidant activity compared to the other solvents (Fig 5.3). The acetone and ethyl acetate root bark extracts had many more compounds with antioxidant activity than the other extracts (Fig 5.4). Based on R<sub>f</sub> values, the antioxidant compounds were different from the antibacterial compounds.

## 5.5 Conclusion

The antiviral activity of extracts of *C. paniculatum* against FHV-1 was meaningful. For an extract that is a crude product, a 3.6 log<sub>10</sub> reduction is a promising result. The acetone extract of the leaves inhibited

viral growth by 3 log<sub>10</sub>. The EC<sub>50</sub> value obtained (2.8 µg/ml) for FHV-1 was close to that obtained by Asres *et al.* (2001) of 3.0 µg/ml against HIV-2. The acetone and water extracts had antiviral activity, hence it was decided to use these two solvents for bulk extraction.

The MIC values of the leaf extract against *S. aureus*, *E. faecalis*, *P. aeruginosa*, and *E. coli* ranged between 0.156 to 2.5 mg/ml. This was close to the values (0.8 - 1.6 mg/ml) found by Eloff (1999) of *C. paniculatum* growing in the Lowveld Botanical Garden. MICs of the root bark extracts ranged between 0.31 and 1.25 mg/ml. These values were higher than those obtained for some other members of the Combretaceae family (Eloff, 1999). The MIC values obtained by Kotze and Eloff (2002) while investigating *C. microphyllum* were in the range of 0.29 to 1.25 mg/ml which was in the range obtained for *C. paniculatum*.