Chapter 9

Isolation and determination of chemical structure of compounds from *Podocarpus henkelii* Stapt ex Dallim. & Jacks

9.1. Introduction

After solvent-solvent fractionation of the *P. henkelii* acetone leaf extract, and identification of fractions with activity against bacterial and fungal pathogens, the next step was to isolate and identify the bioactive compounds. The chemical structure of a compound can be elucidated using a combination of different techniques such as nuclear magnetic resonance (NMR) spectroscopy, mass spectrometry (MS), ultraviolet (UV) and infrared (IR) spectrometry. The information acquired from the various techniques can then be put together to obtain key structural facts.

The approach in acquiring these data is of paramount importance. A good start will be to obtain information on the molecular formula from the high resolution mass spectrum, or from the molecular ion mass and the number of signals present in the carbon and proton NMR spectra. One can then proceed to determine the number of double bonds and/or rings present by calculating the degree of unsaturation and thereafter that of the functional groups and other molecular fragments present from the ^{1}H and ^{13}C NMR spectra. This information is then assembled, wrong structures eliminated and the correct structure is verified by reanalyzing the NMR spectra against the proposed structure.

9.2. Materials and Methods

9.2.1. Column chromatography

Following bioassay-guided fractionation, the ethyl acetate, carbon tetrachloride and chloroform fractions containing the highest numbers of antibacterial and antifungal compounds were combined and dried under a stream of air. The fraction (mass/g) was mixed with silica gel as stationary phase. The silica gel column (60cm x 5cm) as eluted in a gradient system of chloroform: methanol (9:1) to separate the bioactive compounds. Fractions (109, of 30 ml volume)

were collected and combined to produce four fractions based on similar compounds noted in TLC fingerprints. Compound 2 crystallized out of the eluting solvent while compounds 1 and 3 were obtained on final purifications with equal volume of hexane: ethyl acetate (1:1).

9.2.2. Structural elucidation

The compounds isolated were subjected to instrumental analysis. The compounds were dissolved in methanol, chloroform and dimethyl sulfoxide depending on solubility of the compound for analysis. The structures of the three compounds were elucidated using ^{1}H NMR, ^{13}C NMR spectroscopy and mass spectrometry.

9.3. Results and Discussion

Bioassay-guided fractionation of the acetone leaf extract of *Podocarpus henkelii* using column chromatography led to the isolation of three biflavonoids. The three compounds are structurally related C-C linked biflavonoids. The NMR spectra indicated two flavone units linked through the C-3 of a flavone ring to the C-8 of the second flavone. This class of compound consists of three ring systems: A, B, and C. Ring B, which has a hydroxyl substitution at C-4, often gives a typical 4 peak pattern of two doublets (AA'BB' system) with a characteristic coupling constant; this pattern was clearly shown in C-3, C-5, C-2 and C-6 with a coupling constant of 9 Hz at ring IIB. The protons at H-6 and H-8 in ring A, which are metacoupled also showed a characteristic coupling constant of 2.1 Hz. The isolated compounds (Figure 9.1) were identified as 7,4'7'',4'''- tetramethoxy amentoflavone (**1**), isoginkgetin (**2)** and Podocarpus flavones-A (**3)** based on comparison of NMR and MS data with literature values (Krauze-Baranowska *et al*., 2004; Amaro-Luis *et al*., 2008). The NMR and MS date is included in the appendix.

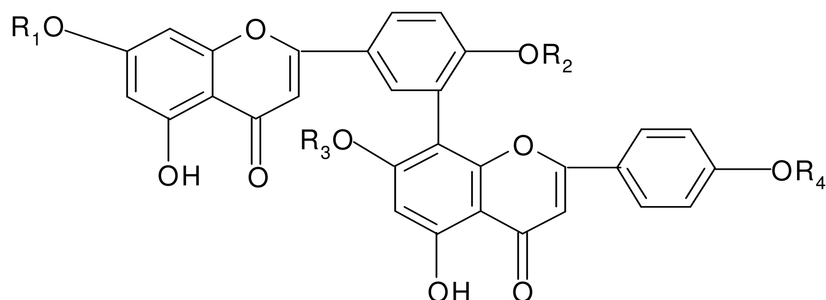

Figure 9.1: Structure of compounds isolated from *Podocarpus henkelii*

Compound 1: $R_1 = R_2 = R_3 = R_4 = CH_3$ 7', 4', 7'', 4''', tetramethoxy amentoflavone

Compound 2: $R_1 = R_3 = H$, $R_2 = R_4 = CH_3$ isoginkgetin

Compound 3: $R_1 = R_2 = R_3 = H$, $R_4 = CH_3$ podocarpusflavone –A

9.4.Conclusion

Three biflavonoids were isolated from *Podocarpus henkelii* and the structures elucidated using data obtained from ^{1}H NMR, ^{13}C NMR and MS analysis. The isolated compounds were identified as 7,4'7'',4'''- tetramethoxy amentoflavone (**1**), isoginkgetin (**2**) and Podocarpusflavones-A (**3**). Available reports indicate the presence of Podocarpusflavone-A in every species of *Podocarpus* so far investigated, except *P. latifolius*. This study represents the first report of isolation of compounds from *Podocarpus henkelii,* as well as the presence of *Podocarpus* flavones-A in this species.

Chapter 10

Biological activity and toxicity studies of isolated compounds from *Podocarpus henkelii* Stapf ex Dallim. & Jacks.

10.1. Introduction

In nature, different types of plants produce certain chemicals (phytoalexins) that are naturally toxic to microorganisms. These chemicals produced by plants play an essential role in the natural defence and well-being of plants, and belong to a wide range of classes, which include the flavonoids and isoflavanoids (Smith, 1996). Flavonoids can be classified into flavanones, flavones, flavonols, and biflavones (Beecher, 2003). Biflavonoids are linkages of flavone–flavone, flavanone–flavones or flavanone–flavanone subunits. Naturally occurring flavonoids are polyphenolic compounds, which can be found in different parts of plants such as flowers, fruits, nuts, seeds, stems and vegetables. They can also be found in wine, honey and commonly consumed beverages such as tea (Grange and Davey, 1990; Middleton and Chithan 1993).

Apart from the phytonutritional role of flavonoids in providing beneficial health effects by the alteration of various metabolic processes, these classes of compounds have been acclaimed for their neuroprotective effect (Kang *et al*., 2005), antiparasitic activity (Mbwanbo *et al.,* 2006), protective effect against DNA damage and lipoperoxidation (Yamaguchi *et al.,* 2005), antiviral activity (Lin *et al.,* 1997; Miki *et al.,* 2007), antimicrobial activity (Lin *et al*., 2001; Xu and Lee, 2001; Yenjai *et al*., 2004*;* Martini *et al*., 2004), anti-inflammatory activity (Selvam and Jachak, 2004), antioxidant activity (Cardoso *et al.,* 2005) and many more.

The biflavonoids Podocarpusflavone-A and isoginkgetin have previously been isolated from *Podocarpus neriifolius* D.Don (Podocarpaceae) (Rizvi *et al.,* 1974). The compound 7', 4', 7'', 4''', tetramethoxy amentoflavone on the other hand has been isolated from *Dacrydium cupressinum* and *Araucaria cooki* (Hodges, 1965). Isoginkgetin has been reported to be less toxic to rat skeletal muscle myoblasts *in vitro* (Weniger *et al*., 2006) in addition to its inhibition of tumour cell invasion by regulating phosphatidylinositol 3-kinase/Akt -dependent matrix metalloproteinase-9 expression (Yoon *et al*., 2006). It has also been found to possess an inhibitory effect on pre-mRNA splicing (O'Brien *et al*., 2008) and neuroprotective effects *in vitro* (Kang *et al.,* 2005).

The presence of diverse molecules represented in the class of biflavonoids and their symmetrical or asymmetrical nature, offers a huge leeway for manipulation by synthetic chemists to further potentiate the biological activity of these useful classes of compounds. Despite the promise and potential therapeutic relevance of this class of compounds, very few biflavonoids have been investigated either for their biological activity, toxicity or as leads for the development of new drugs.

To date no information on antimicrobial activity of these biflavonoids is available. Hence, this study was aimed at evaluating the antibacterial and antifungal activity of the compounds, namely isoginkgetin, Podocarpusflavone-A and 7', 4', 7'', 4''', tetramethoxy amentoflavone isolated from *Podocarpus henkelii*. Their cytotoxic effects on the viability of Vero, bovine dermis and CRFK cells were also assessed in order to determine their selective inhibitory activity, as well as their ability to cause genetic damage with resultant gene mutations as measured by the Ames test.

10.2. Materials and Methods

10.2.1. Determination of minimum inhibitory concentration (MIC) of isolated compounds against bacterial pathogens

The serial microtitre dilution method described by Eloff (1998b) was used to determine the minimum inhibitory concentration (MIC) values of the isolated compounds. The activity of the isolated compounds (1 mg/mℓ) dissolved in DMSO was evaluated against test pathogens (section 3.9.1) using the dilution procedure described in section 3.9.1.1. After incubation for 10 h, 40 µℓ of 0.2 mg/ml INT was added and the plates were further incubated for 2 h. MIC readings were recorded after 12 and 24 hours incubation. Solvent controls and 0.1 mg/mℓ of the standard antibiotic gentamicin (50 mg/mℓ, Virbac) were included in each experiment.

10.2.2. Determination of minimum inhibitory concentration (MIC) of isolated compounds against fungal pathogens

In the antifungal bioassay, the method described by Eloff (1998b) and modified by Masoko *et al*. (2008) using Sabouraud Dextrose (SD) broth as nutrient medium was used to test the activity of isolated compounds. The activity

of the isolated compounds were tested at 1 mg/ml dissolved in DMSO against test pathogens (section 3.9.2.1) using the method described in section 3.9.2.2. INT was used as an indicator of growth. A solvent control and amphotericin B (0.08 mg/ml), a standard antifungal agent, was included as a positive control.

10.2.3. Virucidal assay

The virucidal activity of isolated compounds was evaluated using the method described by Barnard *et al.* (1992) with slight modifications as described in section 3.12.2. The activities of the compounds were tested at 2 mg/mℓ since this concentration did not show toxic effects on cells in the cytotoxicity assay. Antiviral activity was evaluated by the ability of the compound to reduce viral-induced CPE by microscopic examination as well as the MTT colorimetric assay. Antiviral activity was determined as described in section 3.12.2. Negative controls comprised non-infected and untreated cells while positive controls consisted of infected and untreated cells.

10.2.4 Attachment assay

The ability of the viruses to attach to the host cell was tested using the method of Barnard *et al*. (1993) with slight modifications as described in section 3.12.3. Antiviral activity by CPE reduction and MTT assay was determined as previously described (section 3.12.2). Negative controls comprised non-infected and untreated cells while positive controls consisted of infected and untreated cells.

10.2.5. Cytotoxicity assay using MTT

The cytotoxic effects of compounds at 2 mg/ml dissolved in DMSO were tested against the Vero monkey kidney cell line, CRFK cells and bovine dermis cells as described in section 3.10. Compounds 1 and 2 (2 mg) were dissolved in 0.1 ml DMSO to produce a stock concentration of 20 mg/ml solution. Compound 3 was not isolated in sufficient quantity to allow testing for cytotoxicity. The cytotoxicity was expressed as 50% cytotoxic concentration (CC_{50}) of substances to inhibit the growth of cells by 50%, when compared to untreated cells, calculated from the linear regression equation. Berberine chloride (Sigma) was used as a positive control; wells containing only cells without compound treatment were the negative control and a solvent control was also included. For the purpose of calculating selectivity index (SI), cytotoxicity values greater than 1000 µg/mℓ were taken as being 1000. Selective activities of the compounds were calculated as follows:

Selectivity index (SI) = CC_{50} / MIC

10.2.6. Genotoxicity testing of isolated compounds

The compounds 1 and 2 were investigated for their potential mutagenic effect using the plate incorporation procedure as described in section 3.11. The positive control used was 4-nitroquinoline-1-oxide (4-NQO) at a concentration of 2 µg/mℓ.

10.3. Results and Discussion

10.3.1. Antibacterial activity of compounds

The identified biflavonoids were assayed for antibacterial activity against two Gram-positive and two Gram-negative bacterial strains. Results of antibacterial activity of compounds against test organisms are represented in Tables 10.1a and 10.1b. There were no changes in MIC values with an extended time of incubation, suggesting that the activity was bactericidal rather than bacteriostatic. The Gram-positive organisms were more sensitive to test compounds than their Gram-negative counterparts. This finding is consistent with reports ascribing the effectiveness of antimicrobial agents against Gram-positive bacteria to the porous nature of the outer peptido-glycan layer (Nostro *et al*., 2000; Hodges, 2002). However, with flavonoids, two factors have been reported to be important in their antibacterial activity, namely the lipophilicity of the compounds and the presence of a hydroxyl substitution on the phenolic ring, especially at the 4th and the 5th positions. The lipophilic nature, which is enhanced by increasing the number of methoxy substitutions, is responsible for the trapping of flavonoids in the lipophilic cell wall of the bacteria (mainly Gram-negative). This possibly explains why compound **1** is the least active of the isolated biflavonoids against all the tested pathogens with MIC range of 130-250 ug/mℓ. The MIC values of compounds **2** and **3** ranged between 60 and 250 ug/mℓ. Compound **2** was the most active against all the test pathogens with good activity against *S. aureus* and *E. faecalis* (MIC = 60 ug/mℓ) and a higher selectivity index value (Table 10.1b). The three compounds have a 5th hydroxy substituent, which possibly explains some measure of activity. Compound **3**, which had the highest number of hydroxy substituents, had a broader spectrum of activity than the other compounds against *E. faecalis* and *P. aeruginosa* (MIC = 60 ug/mℓ). Previous reports (Lin *et al*., 2001) showed that some biflavones with hydroxyl substituents were completely inactive against *M. tuberculosis*. In this study, the high number of hydroxyl substitution of compound **3** may be responsible for the uptake of this compound by the organisms. This

finding is in agreement with previous reports ascribing structural activity relationships of flavonoids with antibacterial activity (Cushnie and Lamb, 2005).

Table 10.1a. Minimum inhibitory concentration values (ug/mℓ) of isolated compounds against two Gram-positive and two Gram-negative bacteria after 12 and 24 h incubation

Organism	Time(h)	C1	C2	C3	Gentamicin
S. aureus	12	130	60	130	3
E. faecalis		250	60	60	
E. coli		250	130	250	6
P. aeruginosa		250	130	60	
S. aureus	24	130	60	130	3
E. faecalis		250	60	60	
E. coli		250	130	250	6
P. aeruginosa		250	130	60	

Table 10.1b. Selectivity index values of compounds against bacterial pathogens after 12 and 24h incubation

		SI		
	Time (h)	C1	C2	C3
S. aureus	12	7.69	16.67	7.69
E. faecalis		4.00	16.67	16.67
E. coli		4.00	7.69	4.00
P. aeruginosa		4.00	7.69	16.67
S. aureus	24	7.69	16.67	7.69
E. faecalis		4.00	16.67	16.67
E. coli		4.00	7.69	4.00
P. aeruginosa		4.00	7.69	16.67

However, these factors do not completely explain the activity of biflavonoids because Lin *et al.* (2001) found that the methylation or acetylation of these compounds caused no significant change in their activity in that study. It may therefore be possible that the broad spectrum of activity observed with compound **3** may be associated with multiple effects rather than with a specific cellular target.

10.3.2. Antifungal activity of compounds

The activities of the test compounds against fungal pathogens are represented in Tables 10.2a and 10.2b. The trend of activity did not follow the pattern observed in the antibacterial studies. The best antifungal activity was obtained with compound **2** against *A. fumigatus* and *C. neoformans* with MIC of 30 ug/mℓ and excellent selectivity index values of greater than 30. A similar result was obtained for compound **1** against *A. fumigatus*. Compound **3** was less active against the test fungal pathogens with MIC ranging between 130 and 250 ug/mℓ.

Antimicrobial activity exhibited by naturally occurring flavonoids is attributed to the presence of a phenolic group, and the addition of more such groups might potentiate the activity (Harborne and Williams, 2000). However, a study by Picman *et al*. (1995) indicated that increasing the number of hydroxyl, methoxyl or glycosyl substituents resulted in a steady loss of antifungal activity. This observation may possibly explain the low activity exhibited by compound **3** in this study. Other reports (Gafner *et al.,* 1996) suggest that the organism *Verticillium albo-atrum* used by Picman and co-workers (1995) may be exceptional in its response to hydroxyl/methoxyl substitution. Although the fungal pathogens used in that study were plant pathogens, it is not clear whether the response of the pathogens used in this study were influenced by such substitutions. It may be likely that the structure-activity relationship of antifungal compounds could possibly be associated with multiple factors, unlike in bacteria where cell wall interactions are most critical. Variation in time interval and susceptibility of *C. albicans* to compound **1** was observed. This difference in time of incubation suggests that *C. albicans* might have overcome the antifungal effect of compound **1** and was only susceptible at higher concentrations. This observation may suggest a possible fungistatic effect of compound **1** on *C. albicans* after 24 h of incubation. It is also noteworthy that the very slight change in MIC with prolonged time of incubation suggests that the antifungal effect of the compound is long lived.

Table 10.2a. Minimum inhibitory concentration values (ug/mℓ) of compounds against selected fungal pathogens after 24 and 48 h incubation

Organism	Time (h)	C1	C2	C3	Amp-B
C. albicans	24	130	250	250	40
A. fumigatus		30	30	250	80
C. neoformans		130	30	130	20
C. albicans	48	250	250	250	
A. fumigatus		30	30	250	
C. neoformans		130	30	130	

Amp-B = Amphotericin B

Table 10.2b. Selectivity index values of compounds against fungal pathogens after 24 and 48h incubation

		SI		
	Time (h)	C1	C2	C3
C. albicans	24	7.69	4.00	4.00
A. fumigatus		33.33	33.33	4.00
C. neoformans		7.69	33.33	7.69
C. albicans	48	4.00	4.00	4.00
A. fumigatus		33.33	33.33	4.00
C. neoformans		7.69	33.33	7.69

10.3.3. Antiviral activity of compounds

In the virucidal assay, compounds 1, 2 and 3 were incubated with FHV-1, LSDV, CDV and CPI-2 prior to inoculation onto cells, while in the attachment assay, cells were infected with virus prior to addition of test compounds for 1, 2 and 3 hours. In the virucidal assay, viruses were able to induce cytopathic effect when observed by microscopic examination in cells following exposure to test compounds at 10^{-1} dilution prior to inoculation onto cells, suggesting a non-cidal effect on test pathogens. A similar lack of activity of test compounds was observed in the attachment assay. Amentoflavone isolated from the ethanol extract of *Selaginella sinensis* showed potent antiviral activity against respiratory syncytial virus (RSV), with an IC_{50} of 5.5 µg/ml (Ma *et al*. 2001).

Various factors such as lipophilicity and the presence of a hydroxyl substitution on the phenolic ring for antibacterial activity of naturally occurring flavonoids have been suggested. In this study however, the presence or lack of substitution did not seems to potentiate the activity of the isolated compounds. The lack of activity may, in part, relate to the type of viruses used in the study where structural activity relationships are multifactorial rather than targeting a single component.

10.3.4. Toxicity studies of compounds

The mutagenic properties of organic substances, whether synthetic or natural, can be tested using the Ames test (Ames *et al*., 1975). The Ames test is based on a short-term bacterial reverse mutation assay aimed at detecting ranges of chemical substances capable of producing genetic damage with resultant gene mutations. The results from the Ames test performed on the isolated compounds are presented in Table (10.3) as the mean number of revertants per plate in *S. typhimurium* strains TA98 and TA100 ± S.E.M. Compounds **1** and **2** were tested for their potential genotoxic effects in independent repeated assays. Compound **3** was not tested in the Ames test due to the limited quantity isolated. Substances are considered active if the number of induced revertant colonies is twice the number of revertant colonies of the negative control (blank) (Maron and Ames, 1983). None of the compounds investigated was mutagenic in the *Salmonella*/microsome tester strains TA98 and TA100. Flavonoid-induced mutation in the Ames test is reported to more or less match that of structurally related compounds, e.g. naphthalene derivatives, and the pathological consequences of mutation occurring from the eating of flavonoid-containing foods is said to be low

(Habs *et al*., 1984; Bent, 2002). The observations are consistent with findings in this study where compounds tested exhibited no mutagenic effect (Table 10.3).

Table 10.3. Number of his+ revertants in *Salmonella typhimurium* strains TA98 and TA100 produced by isolated compounds

	TA98			TA98		
	No. of colonies			No. of colonies		
	Concentration (µg/ml)			Concentration (µg/ml)		
Compounds	1000	100	10	1000	100	10
C1	23±5.3	28.3±3.2	26.7±3.8	176±31.8	139±2.5	138±67
C2	25.3±4.6	25.3±4.6	25.5±2.1	170.3±225	169±14.6	154±4.4
C3	-	-	-	-	-	-
Spontaneous	19.3±4			152±10		
4NQO	170.3±20			960±35.1		

A similar non-toxic effect was also observed in the cytotoxicity assay when CRFK, Vero and bovine dermis cells were exposed to the test compounds indicating no differences between the three cell lines with regard to their sensitivity to the compounds. Although the influence of structural-activity relationships on cytotoxicity is not well understood, Kuo *et al*. (2008) suggested that OMe and hydroxyl groups in biflavonoids and monoflavonoids play a crucial role in mediating cytotoxic activity. This may possibly explain the observed non-toxic effect of the test compounds.

10.4. Conclusion

Compound C2 was the most active against *E. coli*, *S. aureus, A. fumigatus* and *C. neoformans*, exhibiting both antibacterial and antifungal activity with good selectivity index values. Compound C3 presented a broad spectrum of activity against *E. faecalis* and *P. aeruginosa*. It could therefore be ascertained that the relationship between structures of the compounds and observed biological activity and toxic effect could support the relevance of functional group substitution in the biological activity of biflavonoids. Compounds C1 and C2 showed no deleterious effect in the cytotoxicity assay on various cell lines, and mutagenicity studies indicated the putative non-genotoxic effect of these compounds. Further studies, including those incorporating a metabolic activation step, are necessary to confirm this conclusion. Naturally occurring pure compounds exhibiting good antimicrobial activity which can

selectively kill microorganisms without being significantly toxic to host cells can be a useful tool in evaluating the potential toxic effect of compounds *in vivo*.

Chapter 11

General discussion and conclusion

The aim of this study was to develop a low toxicity plant extract or isolated compound that is effective against selected bacteria, fungi or animal viruses from leaves of the most promising plant and validate its ethnomedicinal use.

11.1. Antibacterial and antifungal activity of different extracts of selected plant species

The antibacterial and antifungal activity of the hexane, DCM, acetone and methanol extracts of seven selected plant species were determined**.** On bioautography, the presence of visible zones of microbial inhibition varied with some extracts. The presence of visible zones of inhibition was influenced by the polarity of solvents used for extraction of plant active constituents. In general, the acetone and methanol extracts of the different plants species had more active compounds (25 each) followed by DCM extracts. This suggests that the active compounds have intermediate polarity or polar characteristics.

The acetone extracts had the best antifungal activity followed by the methanol extracts. Extracts from more than one plant species had activity against all the pathogens. With DCM extracts, activity was only observed against *C.albicans* while with the hexane extracts no substantial activity was observed against any of the pathogens. The activity of the acetone extracts for those plants that had antifungal constituents ranged between 0.16 to 0.08 mg/mℓ against *C. albicans* and *C. neoformans*. The acetone extract of *Podocarpus henkelii* had an MIC of 0.08 mg/mℓ *against C. neoformans* as did the acetone and DCM extracts of *Acokanthera schimperi* against *C. neoformans*. The DCM and methanol extract of *Annona senegalensis* against *A. fumigatus* and *C. neoformans* respectively had MICs of 0.08 mg/mℓ while the DCM extract of *Plumbago zeylanica* had an MIC 0.08 mg/mℓ against *A.fumigatus*. The

activity of the acetone extract is consistent with previous report (Eloff, 1999; Kotze and Eloff, 2002) where acetone was the best extract for antimicrobial activity. The fact that extracts from several plant species had a wide activity against several bacteria and fungi may indicate the presence of a general metabolic toxin. Consequently the cellular toxicity was investigated.

With bacterial pathogens, acetone extracts of the different plants had the best activity with MIC ranging between 0.16 to 0.08 mg/mℓ followed by the DCM and hexane the least. The DCM extract of *Carissa edulis* had the best MIC (0.04 mg/mℓ) against *P. aeruginosa*.

11.2. Determining the cytotoxic effect of the different extracts on different cell types

In general the hexane was the least toxic indicating that highly polar compounds were not toxic, possibly because they could not be absorbed through membranes. The intermediate polarity extracts were generally the most toxic, possibly again because these compounds are better absorbed.

Annona senegalensis and *Acokanthera schimperi* extracts were the most toxic of all the plants evaluated. These plants are toxic to animals and the cytotoxicity is in line with the *in vivo* toxicity. *P zeylanica* and *S. alata* were the least toxic with *C. edulis* and *P. henkelii* having close to the same safety. *C. edulis* fruit are edible and the cytotoxicity data reflect this. Cells were more tolerant to the toxic effect of extracts at 0.01 mg/mℓ and below in those plants that had moderate toxicity.

To determine which cells were the most sensitive, all values for the different plant species and extractants were combined. Of the three cell types used CRFK was slightly sensitive followed by BD and Vero cells. This pattern was valid for all the concentration tested. Vero and CRFK cells are both kidney derived cells, and may therefore be expected to show a similar response to the toxic effect of the extracts, but this was not the case in this study. At the highest concentration (1 mg/mℓ), all the extracts were very toxic to the cells with three exceptions where the methanol extracts were less toxic. At the lowest concentration tested 0.001 mg/ml in most cases there was little cytotoxicity.

Despite the good antibacterial activity recorded, for extracts of many plant species, only *Podocarpus henkelii* and *Plumbago zeylanica* extracts had moderate toxicity on the different cell types used in the toxicity assay. Similarly, the acetone extracts had the best selectivity index value, followed by methanol. The susceptibility of fungal pathogens was generally highest with methanol and acetone extracts of plants against *C.neoformans* respectively. *A.fumigatus* was the most non-susceptible pathogen against hexane, DCM and methonal extracts of the different plant species.

The protective effect of antioxidant constituents in some extracts varied and appears to be influenced by the metabolism of the type of cell in culture. It also appears to suggest that metabolism in kidney-derived cells can be influenced by species variation in the origin of cells. More studies are required to understand the factors responsible for the difference in susceptibility, to provide more insight on possible contra-indication of some herbal remedies in different animal species. The presence of substances in some extracts at low concentrations that induce viable cell proliferation *in vitro* is also worthy of further investigation, which can help understand the positive or negative outcomes of these substances *in vivo*. As a positive outcome, the presence of such substances in certain plants can serve as useful tonics for organ revitalization which can in turn help balance biochemical and physiological events within the body or act as adaptogens that will help enhance a non-specific resistance of the organism to stress factors and thereby promote its adaptation to stressful external conditions (Mowrey, 1998; Antoshechkin, 2001).

11.3. Determining the antiviral activity of different extracts of selected plant species

The antiviral activities against four viral pathogens in the virucidal and attachment assay were determined for the extracts of the different plant species. In general, good selectivity index values were obtained in the virucidal assay: indicating a more potent antiviral activity when compared to the attachment assay. Of the extracts tested in the virucidal assay, four extracts had significant antiviral activity, two of which were different extracts of *Podocarpus henkelii* against two unrelated viruses. The acetone extracts of *Podocarpus henkelii* against CDV and the methanol extract against LSDV had good activity with SI values of 12.01 and 45.61 respectively in the virucidal assay. The hexane extract of *Plumbago zeylanica* on the other hand had good activity against CDV with SI = 3.07 in the virucidal assay. The hexane extract of *Carissa edulis* in the virucidal assay had weak activity against FHV-1 with EC_{50} of 73.17 µg/mℓ and SI 1.22 while the same against CDV exhibited good activity with an EC_{50} 12.37 µg/mℓ and SI 6.14.

Although a different extractant was used than that used by traditional healers, the presence of antiviral compounds in *Podocarpus henkelii* against two unrelated viruses may justify on a the traditional use of related species *Podocarpus latifolius* and *Podocarpus falcatus* in the traditional treatment of canine distemper infection in dogs.

11.4. Selection of plant species for further investigation

From the pool of initially selected plant species, *P. henkelii* was chosen for further investigation based on the following reasons, 1) no compounds have been isolated from this plant and assayed for biological activity, 2) the

acetone and methanol extracts had good antibacterial activity against *E. coli*, *P. aeruginosa* and *E. faecalis,* 3) the acetone extract had excellent antifungal activity against *C. albicans* and good activity against *C. neoformans* and 4) the acetone extract had good to moderate activity against CDV and LSDV in both the virucidal and attachment assays, while the methanol extract was active against LSDV in the virucidal assay.

11.5. Isolation and biological activity of isolated compounds

Using bioassay-guided fractionation, three biflavonoids were isolated from the leaves of *P. henkelii.* ^{13}C and ^{1}H NMR and mass spectrometric data led to the identification of the compounds as 7', 4', 7'', 4''', tetramethoxy amentoflavone (C1), isoginkgetin (C2) and Podocarpusflavone–A (C3). Podocarpusflavone-A and isoginkgetin have previously been isolated from *Podocarpus neriifolius* D.Don (Podocarpaceae) (Rizvi *et al.,* 1974). The compound 7', 4', 7'', 4''', tetramethoxy amentoflavone on the other hand has been isolated from *Dacrydium cupressinum* and *Araucaria cooki* (Hodges, 1965). Podocarpusflavone-A has been found to occur in every species of *Podocarpus* so far investigated, except *P. latifolius.* Reports on biological activity of these compounds are scanty. Isoginkgetin has low toxicity on rat skeletal muscle myoblasts *in vitro* (Weniger *et al*., 2006) in addition to its inhibition of tumour cell invasion by regulating phosphatidylinositol 3-kinase/Akt -dependent matrix metalloproteinase-9 expression (Yoon *et al*., 2006). It has also an inhibitory effect on pre-mRNA splicing (O'Brien *et al*., 2008) and neuroprotective effects *in vitro* (Kang *et al.,* 2005). Apart from the aforementioned, no information is available on the antimicrobial activity of any of these compounds.

Compound C2 was the most active against *E. coli* and *S. aureus* (MIC = 60 ug/mℓ) and SI value of 16.67. The compound was also active against *A. fumigatus* and *C. neoformans* (SI = 33.33) suggesting both antibacterial and antifungal activity with very good selectivity index values. Compound C3 presented a broad spectrum of activity against *E. faecalis* and *P. aeruginosa* with SI values of 4. A less potent activity of the compounds was obtained in both the virucidal and attachment assays against test pathogens, indicating the non-activity of the compounds against tested viral pathogens. The antibacterial and antifungal activity of flavonoids has been reported to be influenced by structural activity relationships (Harborne and Williams, 2000; Cushnie and Lamb, 2005). Structural activity relationships and antiviral activity have also been reported for biflavonoids where methylation of the hydroxyl groups of biflavonoids resulted in diminished activity (Lin *et al*., 1997). It could therefore be necessary to investigate the relationship between structures of the compounds with the aim of synthesizing a nontoxic and still potent derivative.

11.6. The cytotoxicity and genotoxic activity of isolated compounds

The cytotoxicity of the isolated compounds was tested on vero, CRFK and bovine dermis cells using the MTT assay and mutagenic effect in the Ame's test. Compound 3 was not tested due to the small quantity of the compound isolated. In the cytotoxicity assay. Compound C1 and C2 had no deleterious effect on Vero, bovine dermis and CRFK cells and had no mutagenic activity based on the Ames test.

11.7. Evaluating the correlation between antiviral and antimicrobial activity

Correlating toxicity and biological activity of crude extracts, the antimicrobial activity of extracts against test bacterial, fungal and viral pathogens was influenced by the polarity of solvents used for extraction of plant active constituents. The observed activity was consistent against bacterial and fungal pathogens in some plants. In plants such as *C. edulis* and *P. zeylanica* the acetone and methanol extracts were active against all the bacterial and fungal pathogens. On the other hand, only the hexane extracts of these plants had activity in the virucidal assay against CDV with SI values of 6.14 and 3.07 respectively. This suggests that with these plants, antiviral activity is associated with the non-polar constituents rather than with the intermediate and polar constituents. In contrast, good SI values of 12.01 and 45.61 were obtained for the acetone and methanolic extracts of *P. henkelii* against CDV and LSDV respectively. This finding suggests that polarity of solvents used for extraction may not necessarily dictate the presence of antiviral constituents in a given extract. However, the fact that more active constituents are present in the acetone and methanol extracts with significant activity against bacterial, fungal and viral pathogen, suggests that intermediate and polar solvents do extract a range of constituents active against these pathogen. These solvents can therefore be used to extract biological active constituents with a wide range of activity across a broader spectrum.

Many microbes are causative agents for a majority of life threatening diseases worldwide, with significant economic impact to national economies. With the dwindling per capita income of most third world economies, it has become challenging to strike a balance between establishing good health care systems and providing food by sustained growth in the agricultural sector. Coupled with this is the cost associated with treatment of diseases in resource poor settings. The identification and validation, of traditionally use medicinal plants can play a significant role in alleviating health problems within communities; improve livestock production and food security. Plants are a relatively cheap source of biological material consisting of a vast number of metabolites, primary or secondary. These plants can be prepared in different forms for a desired therapeutic effect or for selecting the molecule of desired biological activity. While a desired therapeutic effect is key behind the rationale for its continuous use, a plant may contain substances with effects that may be unfavourable, depending on the solvents used to extract the desired biologically active component.

It would therefore be worthwhile to invest financial resources in investigating the medicinal potential of our rich flora to combat prevailing microbial infections with the hope of finding potential lead targets for use or that can act as templates for the synthesis of effective and cheap antimicrobials or develop low technology extracts based on good science that can address the primary health care needs of poor communities. The use of crude or potentised

extracts, which more often than not retain their therapeutic efficacy as opposed to single lead compounds due to availability and cost in resource poor settings, provides an exciting challenge using plants such as *P. henkelii*.