CHAPTER 7

BIOLOGICAL CHARACTERIZATION OF ISOLATED COMPOUNDS

7.1 Introduction

The biological activity of the isolated compounds were characterised for their individual and combined anti-microbial efficacy and individual anti-inflammatory action. In addition the antiproliferative and cytotoxic effects of the compounds were determined.

7.2 Material and methods

7.2.1 Anti-microbial activity

7.2.1.1 Antibacterial activity (Microplate dilution assay)

The MIC of the isolated compounds were determined using a serial microplate dilution assay (Eloff, 1998) as described in **Section 4.2.3**

7.2.1.2 Antibacterial and antifungal activity (Agar diffusion assay)

The agar diffusion method was used for the determination of antibacterial and antifungal activities of the micro-organisms listed in **Table 7-1**. Approximately 9 ml of Müller–Hinton agar for bacteria and Sabouraud Dextrose Agar for fungi (Oxoid, UK) were poured into Petri dishes (9 cm in diameter) and inoculated with the respective test organisms. Wells (4.0 mm) were punched out of the solid agar using pipette tips, and 1 ml of 50 µg/ml of the test compounds and control antibiotics were placed into each well. Petri dishes were incubated at 37 ^{0}C for 20 h and the average diameter of the inhibition zone surrounding the wells was measured.

Table 7-1: Bacteria and Fungi tested for effficacy in the Agar diffusion assay

Code	Bacteria	HKI Code
BNA	*Bacillus subtilis* ATTC 6633 (IMET) NA	B1
Bas	*Bacillus subtilis* ATTC 6633 (IMET) AS	B2
SA	*Staphylococcus aureus* (IMET 10760) SG 511	B3
EC	*Escherichia coli* SG 458	B4
PA	*Pseudomonas aeruginosa* K 799/61	B9
MS	*Mycobacterium smegmatis* SG 987 (HKI0056)	M2
MV	*Mycobacterium vaccae* IMET 10670	M4
	Fungi	
SS	*Sporobolomyces salmonicolor* SBUG 549	H4
CA	*Candida albicans* BMSY 212	H8
PN	Penicillium notatum	P1

7.2.1.3 Antimicrobial effect of some combined compounds

After determining the MIC of isolated compounds, the compounds that were available in higher quantities were mixed in equal ratios and the MIC of the mixture determined to ascertain whether any synergistic activity of the combined compounds were present. From *C. imberbe*, Compound **1, 4** and **5** were available in high enough quantity and 1 mg/ml of each compound combined as mixtures of **(1+4), (1+5**) and **(1+4+5).** Compounds **6, 7** and **8** were used from *C. padoides* and combined as **(6+7), (6+8**) and **(6+7+8**). All combinations were in a 1:1 ratio. The MIC of all combined compounds was determined using 100 µl of each mixture as described in Section **4.2.3** and compared to the activity of the individual compounds. A decrease in the MIC of the most effective compound in the mixture was accepted as an indication synergistic activity.

7.2.2 Anti-inflammatory activity

This analysis was carried out by the Molecular Natural Product Research group of HKI, Jena, Germany.

The NAD (P)-linked enzyme, 3α-hydroxysteroid dehydrogenase, has been purified to homogeneity from rat liver cytosol (Penning, 1983). This enzyme is known to reduce a variety of 3-ketosteroids, e.g., 5α-dihydrotestosterone (5α-androstan-17β-o-one), 5β-dihydrocortisone (5β-pregnan-17α, 21-diol-3, 11, 20-

trione), to the corresponding 3α-hydroxysteroids and, therefore, plays an important role in cortisone metabolism (Penning, 1983). A surprising property of the purified enzyme is that it is potently inhibited by the major classes of non-steroidal and steroidal anti-inflammatory drugs in rank order of their therapeutic potency (Penning, 1983). A high correlation exists between the logarithm of the concentration of drug required to produce 50% inhibition of the purified 3α-hydroxysteroid dehydrogenase (log IC_{50} value) with the dose required to produce an anti-inflammatory response in man. These observations led to the suggestion that the extent of inhibition of 3α-hydroxysteroid dehydrogenase could be used to predict anti-inflammatory drug potency (Penning, 1983).

7.2.2.1 Preparation of Cytosol

Adult male Sprague-Dawley rats (150-200 g) were killed by cervical dislocation. The livers were excised and homogenized in 3 volumes of 50 mM Tris-HCI of pH 8.6 containing 250 mM sucrose, 1 mM dithiothreitol, and 1 mM EDTA. Homogenates were centrifuged at 100,000xg for 30min; the supernatant (cytosol; i.e., source of 3α-hydroxysteroid dehydrogenase) was used for enzyme assays without further processing.

7.2.2.2 Preparation of Purified 3α-Hydroxysteroid Dehydrogenase

Homogeneous enzyme was prepared according to the method described by Penning, 1983. This enzyme had a final specific activity of 3.58 µmol of 5β-dihydrocortisone reduced/min/mg of protein.

7.2.2.3 Enzyme Assays

The reduction of 5β-dihydrocortisone was monitored by measuring the changes in the absorbance of the pyridine nucleotide at 340 nm. Each assay (1.0 ml) contained the following: 0. 840 ml of H_2O, 0.100 ml of 1 M potassium phosphate buffer (p 6.0), 20 µL of 9 M NADPH, 10 µL of 5 mM 5β-dihydrocortisone, and 30 µL of acetonitrile. The reactions were initiated by the addition of enzyme (30-50 µg of cytosolic protein or 0.6 µg of purified enzyme), and optical density change was followed over a period of 5 minutes. Control incubation experiments by addition of the cytosol in which either the 5β-dihydrocortsone or NADPH was absent, indicated that the presence of both substances was required before the cytosol would promote a change in absorbance at 340 nm.

7.2.2.4 Inhibition Studies

The % inhibition of seven isolated compounds was generated at three different concentrations (30 µg/ml, 3 µg/ml and 0.3 µg/ml). Increasing amounts of the isolated compound was added to the standard assay system, and the concentration of the compound required to reduce the rate of 5β -dihydrocortisone reductions by 50% (IC_{50}) was computed from the resulting ln dose-response curves.**7.2.3**

7.2.3 Antiproliferative and cytotoxicity assay

This analysis was carried out by the Molecular Natural Product Research group of HKI, Jena, Germany.

Isolated compounds were assayed on cell lines K-562 (human chronic myeloid leukaemia) and L-929 (mouse fibroblast) for their antiproliferative effects (GI_{50}: concentration which inhibited cell growth by 50%), and against Hela for their cytotoxicity effects (CC_{50}: concentration at which numbers of cells are destroyed by 50%; used particularly in referring to the lysis of cells). The cells were incubated in 10 different concentrations of each of the target compounds. Fifteen cells of established suspended K-562 (DSM ACC 10) and Hela, and adherent L-929 (DSM ACC 2) cell lines were cultured in RPMI medium. The adherent cells of L-929 and Hela were harvested at the logarithmic growth phase after trypsinization, using 0.25 % trypsin in PBS containing 0.02% EDTA (Biochrom KG Kat.-Nr. L2163). The inherent cells of L-929 were harvested at the logarithmic growth phase after soft trypsinization, using 0.25% trypsin in PBS containing 0.02% EDTA (Biochrom KG L2163). For each experiment with L-929 and Hela approximately 10,000 cells were seeded with 0.1 ml RPMI 1640 (GIPCO BRL 21875-034), containing 25 ug/ml gentamicin sulfate (BioWhittaker 17- 528Z), but without HEPES, per well of the 96-well microplates (K-562: NUNC 163320, L-929, Hela: NUNC 167008). For the cytotoxic assay, the Hela cells were preinoculated for 48 hours without the test substances. The dilution of the compounds was carefully carried out on the monolayer of Hela cells after the pre-incubated time.

Cells of L-929, K-562 and Hela, with the presence the respective compounds, were incubated for 72 hours at 37^0C in a humidified atmosphere and 5% CO_2. Suspension cultures of L-562 in microplates were analysed by an electronic cell analyser system CASY 1 (SCHARFE, Reutlingen, Germany) using an aperture of 150 um. The 0.2 ml content of each well in the microplate was diluted 1:50 with CASYTON (SCHARFE). Every count/ml was automatically calculated from the arithmetic mean of three successive

counts of 0.4 ml each. From the dose response curves the GI_{50} values were calculated with CASYSTAT. The GI_{50} value was defined as the 50 % intersection line of the ln concentration-response curve, determined by the cell count/ml as compared to the control. The essential parameters for the estimation of growth inhibition and for change in diameter distribution curve were expressed as diagrams. The monolayer of the adherent L-929 and Hela cells were fixed by glutaraldehyde and stained with a solution of methylene blue. After gently washing, the stain was eluted by 0.2 ml of 0.33 N HCl in the wells. The optical densities were measured at 630 nm in a microplate reader.

7.3 Results and Discussion

7.3.1 Antibacterial activity (Microplate dilution assay)

The MIC result of the isolated compounds tested using the microplate dilution assay are presented in **Table 7-2**

Table 7-2: MIC of compounds isolated from *C. imberbe* and *C. padoides* [*S. aureus* (SA), *E. faecalis* (EF), and *E. coli* (EC), *P. aeruginosa* (PA)]

Compounds	MIC (µg/ml)			
	SA	*EF*	*EC*	*PA*
C. imberbe				
1	130	130	20	>300
2	90	20	>400	200
3	130	130	20	>300
4	60	> 300	20	>300
5	60	> 300	20	>300
C. padoides	*SA*	*EF*	*EC*	*PA*
6	60	>300	60	>300
7	30	130	60	>300
8	>300	>300	60	>300
Gentamycin	5.8	5.8	5.8	5.8

All compounds, except for Compounds **2** and **8**, showed the highest antibacterial activity against *E.coli* and *S. aureus* and were all either poorly active or inactive against *P. aeruginosa.* Compound **2** was ineffective against *E.coli* and Compound **8** against *S. aureus.* Compound **2** was the only compound with high effectivity (20 µg/ml) against *E. faecalis*; all other compounds were either poorly active (>100 µg/ml) or inactive (>300 µg/ml). The high activity of Compounds **1, 3, 4** and **5** from *C. imberbe* against *E. coli* is in contrast to the observation that *E. coli* was resistant to other pentacyclic triterpenes isolated from the same plant species (Katerere *et al.*, 2002). Pentacyclic and tetracyclic triterpenes are known for their action as molluscides, particularly in their monodesmosidic form (Marston and Hostettmann, 1985). *C. molle* is known for its molluscidal constituent, mollic acid, which has been recommended for use in rural Africa to control schistosomiasis (Rogers, 1996). Arjunolic acid and arjungenin, arjunglucoside pentacyclic triterpenes have been isolated from C. molle (Panzini I., 1993).

Apart from this work, there are few data on the antimicrobial potential of the isoprenoid constituents of Combretaceae. Eloff (1998) reported preliminary data on crude extracts of *C. imberbe and C. padoides* against microbial culture. Martini and Eloff (1998) also showed that crude extracts of *C. erythrophyllum* are active against microbial cultures (*S. aureus and E. faecalis*) and indicated that their results potentially support the use of this plant in traditional medicine for relieving symptoms that appear to be caused by infective agents e.g. bloody diarrhoea, wounds and conjunctivitis (Gelfand *et al*., 1985). The present results further confirm the activity of the constituents of *Combretum species* against bacteria and justify the potential use of *C. imberbe* in folk medicine, as well as expand our knowledge on the antibacterial activity of *C. imberbe* and *C. padoides.* Some of the compounds isolated are candidates for further work to evaluate their therapeutic potential.

7.3.2 Antimicrobial activity (Agar diffusion assay)

The agar diffusion method was used as an alternative method to evaluate the antibacterial effect of the isolated compounds as well as to test their antifungal activity. The results are presented in **Table 7-3.**

Table 7-3: Zone of inhibition of compounds isolated from *C. imberbe* and *C. padoides* against several bacterial and fungal organisms (*B. subtilis* ATTC 6633 (IMET) NA (B1), *B. subtilis* ATTC 6633 (IMET) NS (B2), *S. aureus* (IMET 10760) SG511 (B3), *E. coli* SG 458 (B4), *P. aeruginosa* K 799/61 (B9), *M. smegmatis* SG 987 (HKI0056) (M2), *M. vaccae* IMET 10670 (M4) H4 H8 & P1

Compounds	Zone of Inhibition (mm)									
	B1	B2	B3	B4	B9	M2	M4	H4	H8	P1
C. imberbe										
1	22		23/28p	0	14p		26	0	0	0
3	10	10	-	0	0	-	0	0	0	0
4	11	10	11	0	0	-	12p	0	0	0
5	21	21/26p	-	12p	17p	-	24	0	0	0
C. padoides	B1	B2	B3	B4	B9	M2	M4	H4	H8	P1
6	14/17.5p	22/26.5p	-	0	12p	-	18	0	26p	0
7	11	10	11	0	0	-	12p	0	19p	0
8	0	0	'	0	0	-	0	0	0	0

p = partial inhibition (-) Test not done on these organisms because of low quantity of the compounds

Several compounds inhibited bacterial and fungal growth. The zones of inhibition for the active compounds were in the range 10-26 mm. Generally, in this assay all the isolated compounds had a better activity against bacteria than fungi. For the compounds isolated from *C. imberbe*, Compound **1** had a good inhibitory activity against *B. subtilis* (22 mm), *S. aureus* (23 mm and a partial inhibition of 26 mm), *and M. vaccae* (26 mm). Compound **5** also had a relatively good activity against *B. subtilis (*21 mm*), S. aureus* 21 mm and a partial inhibition of 26 mm and *M. vaccae* (24 mm). Earlier studies (Katerere *et al.,* 2002) have shown that pentacyclic triterpene from *C. imberbe* has activity against *M. fortuitum*.

Compounds isolated from *C. padoides* indicated a good activity as well. Compound **6** showed an inhibitory effect against *B. subtilis (*22 mm and a partial inhibition of 26.5 mm*), M. vaccae* (18 mm) and a partial inhibition of 26 mm against *C. albicans*.

It has been shown that extracts of some *C.* species (*C. glutinosum, C. hispidum, C. molle* and *C. nigricans)* have antifungal effect against dermatophytes as well *C. albicans* (Baba-Moussa *et al.*, 1999). This report proposed that tannins and saponins might be responsible for this activity that might explain the good activity of Compound **5** and **6** being isoprenoid glycosides.

All the organisms were resistant to Compound **8** that indicated that steroid glycosides might not have anti-microbial activity.

Comparison of the MIC and the zone inhibition results did not appear to indicate similarity in response against the same organisms. Despite high effectivity (low MIC) of Compounds **1, 3, 4, 6, 6** & **8** against *E. coli* on the microplate dilution assay, no zones of inhibition was observed in the Agar diffusion assay for all of these compounds, except Compound **5** that had a partial zone of inhibition. Compound **1** showed a wide zone of inhibition against *S. aureus* yet only had a moderate MIC, whereas Compounds **4** & **7**, which had a lower MIC than Compound **1**, had a smaller zone of inhibition. The diference could be in the rate of diffusion of the various compounds in agar medium. No activity was observed against *P aeruginosa* for all isolated compounds using the microplate dilution assay, whereas partial zones of inhibition were observed for Compounds **1**, **5** and **6**.

7.3.3 Combined antimicrobial effect of mixtures of isolated compounds

The synergistic and antagonistic effect of Compounds **1, 4 & 5** from *C. imberbe* and Compounds **6**, **7** & **8** from *C. padoides* are shown in **Table 7-4**

The MIC values obtained with the individual compounds are consistent with the original microplate dilution assay using the isolated compounds. Synergistic activity were observed with compound mixtures **1+4+5** of *C. imberbe* against *S. aureus* and compound mixtures **6+7** and **6+8** of *C. padiodes* against *S. aureus* and *E. coli* . The antibacterial effect of compound mixtures were regarded as synergistic if the MIC value achieved with the combination was less than lowest MIC value of any of the individual compounds. An antagonistic effect, where the MIC of the compound mixtures was larger more than two fold higher than the lowest MIC of the individual components, was noted with mixture **1+4** against all bacterial species; and mixtures **1+5** and **1+4+5** against *E. faecalis* and *E. coli*.

Table 7-4: MIC (µg/ml) values of individual and mixtures of isolated compounds

Compound Mixtures	Minimum Inhibitory Concentration (µg/ml)		
	S. aureus	*E. faecalis*	*E. coli*
C. imberbe			
1	125	125	16
2	93	23	>250
3	125	125	16
4	63	>250	16
5	63	>250	16
1+4	>250	>250	31
1+5	63	>250	63
4+5	63	>250	63
1+4+5	31	>250	>250
C. padoides	*S. aureus*	*E. faecalis*	*E.coli*
6	63	>250	63
7	31	125	63
8	>250	>250	63
6+7	16	125	31
6+8	31	>250	31
7+8	31	>250	63
6+7+8	31	>250	63

In contrast to single remedy product (Harris 2003) explans that combination preparation are difficult to evaluate scientifically. The synergistic effect of combinations of isolated compounds found in the current study is in contrast to the views by aromatherapists who maintain that isolated compounds do not exhibit synergy (Harris, 2003). Harris (2003) explained that this argument is not borne out by research, that it is possible to achieve synergism through mixing isolates; natural or synthetic. One such study (Didry et al., 1994) tested the effects of combination of components such as thymol, eugenol, carvacrol and cinnamaldehyde on oral bacteria. Synergistic effects were found between certain blends such as thymol and caevacrol, eugenol and thymol, eugenol and carvacrol. Delaquis (2002) also found that when the principal isolates of *Anethum graveolens* (limonene and carvone) were blended together and then enriched with either terpene, their antimicrobial effects were significantly more active than whole oil. It was concluded that

using the isolates as opposed to the whole oil would allow for preparations of constant chemical composition and confirmed synergy.

Antagonism between isolated components has previously been reported by (Fanaki, 1997) when testing a frequently used antimicrobial preparation of essential oil components (containing anethole, borneol, camhene, 1,8-cineole, fenchone and pinenes) against multi-resistant bacterial strains. The finding demonstrated that the combination was less effective than the combined activity of the isolates when tested individually.

It is important to understand that isolates when combined may produce synergistic effect or antagonistic effect depending on the ratio of their combination. Studies by Low *et al.,*1974 on the interactions between citronellal and citronellol showed that as long as the naturally occurring ratio of these two substances was respected, a similar synergy will be obtained if the components were synthetically produced. Other factors that might not have been scientifically proven might also be involved.

7.3.4 Anti-inflammatory activity

The percentage inhibition of purified NAD (P)-linked 3α-hydroxysteroid dehydrogenase of seven isolated compounds at three different concentrations (30 µg/ml, 3 µg/ml and 0.3 µg/ml), IC50 and HKI class are given in **Table 7-.5**.

Table 7-5: Anti-inflammatory activity of compounds isolated from *C. imberbe* and *C. padoides.*

Compounds	% Inhibition 3α-hydroxysteroid dehydrogenase			IC_{50}	HKI Class
	30 µg/ml	3 µg/ml	0.3 µg/ml		
C. imberbe					
1	88	84	0	0.3	3
3	85	36	0	0.3	3
4	59	10	0	7.8	1
5	63	17	13	9.5	1
C. padoides	**30 µg/ml**	**3 µg/ml**	**0.3 µg/ml**	**IC_{50}**	**HKI Class**
6	41	0	0	13	0
7	25	0	0	13.5	0
8	94	72	0	5.1	1

Not active (0), active (1), more active (2), highly active (3)

Inhibition of NAD (P) linked 3α-hydroxylsteroid dehydrogenase of rat liver cytosol is correlated with anti-inflammatory activity in man (Penning, 1983). Compounds **1** and **3** isolated from *C. imberbe* had an anti-inflammatory effect with IC_{50} of 0.30 µg/ml each. These compounds fall in Class 3 of the Hans-Knöll institut (HKI) standards, indicating very good anti-inflammatory activity. Compounds **4** and **5** from *C. imberbe* and Compound **8** from *C. padoides* had moderate anti-inflammatory effect and are considered as Class 1 anti-inflammatory compounds.

7.3.5 Anti-proliferative effect and Cytotoxicity

The antiproliferative and cytotoxic efficacy of compounds **1-8** was tested *in vitro* against (L-929, k-562) and (Hela) cell lines, respectively. The antiproliferative and cytotoxic data are shown in **Table 7-6.**

Table 7-6: Anti-proliferative and cytotoxic effect of compounds isolated from *C. imberbe* **and** *C. padoides.*

Compounds	Antiproliferative effect (µg/ml)		Cytotoxic effect (µg/ml)
	(L-929) GI_{50}	(K-562)GI_{50}	(Hela) CC_{50}
C. imberbe			
1	32,9	28,1	34,9
2	-	-	-
3	9	8,7	10,5
4	> 50	> 50	> 50
5	16,5	13,5	17,5
C. padoides	L-929) GI_{50}	(K-562)GI_{50}	Hela) CC_{50}
6	> 50	> 50	> 50
7	> 50	> 50	44,7
8	> 50	> 50	> 50

Antiproliferative activity not done with this compound because low quantity.

Most of the compounds particularly those isolated from *C. padoides* had slight anti-proliferative and cytotoxic effect. In general the compounds had a greater anti-proliferative than cytotoxic effect. Compounds **3** and **5** showed a strong antiproliferative activity against both L-929 and K-562 cell lines Compound **3** showed a moderate cytotoxic effect of CC_{50} =10.5 µg/ml against Hela cells.

There have been a very few reports on the anti-proliferative and cytotoxic effect of the constituents of Combretaceae. This study indicates that some members of the Combretaceae may have antiproliferative and cytotoxic constituents. This aspect may justify further research.

7.3.5 Structure activity relationship of isolated compounds

The level of antibacterial activity of the various isolated compounds was compared with the structure of each compound to determine the structural: activity relationship of each compound. Generally, the aglycones had a better antibacterial activity against *S. aureus* and *E. coli* than the glycosides (**Table 7-2**). It is also proposed that the antibacterial activity of the aglycones increase with respect to the numbers of OH groups present. This may explain why Compound **4** (0.060 mg/ml against *S. aureus*) with two OH groups has a lower MIC than Compound **3** with only one OH group.

Compounds **5** and **6** are glycosides with MIC of 0.016 mg/ml against *E. coli*, which is the same as the aglycone of these compounds hence suggesting that the sugar unit may not play any role in the activity of the compounds against *E. coli*. A very poor activity against all the organisms was observed with Compound 8 that is a glycoside with an aglycone that does not have any OH group. This further supports the observation that the presence of OH influences antibacterial effect. This is in contracst to earlierstudy wihich shows that the hydorxyl group position in the triterpene skeleton has an influence in the activity of triterpene and that the increase in the number of hydroxyl group dreceases the antibacterial activity of a triterpene (Djoukeng *et al*., 2005).

Structure activity relationships as observed in the current study may help natural product chemists to arrive at new biologically active derivatives through synthesis of compounds that might have pharmaceutical properties.

7.4 Summary

Most of the compounds isolated had a broad-spectrum antibacterial activity against Gram-positive and Gram-negative pathogens with an MIC range of 20 to 250 µg/ml for Gram-positive bacteria and 20 to 250 µg/ml for Gram-negative bacteria. In the agar well diffusion assay, Compound **1** had the highest activity against M. viccae IMET 10670 with inhibition zone of 26 mm. Compound **5** also had a good activity against *M. vaccae* with zone of inhibition of 22 mm. Compound **6** exhibited fungicidal activity (18 mm) against *S.*

salmonicolor and a partial activity of 26 mm against *C. albicans.* This confirms that components of Combretaceae have both antifungal and antibacterial activity.

There was synergistic effect as well as antagonistic effect amongst some mixtures of the isolated compounds. Compounds isolated from *C. padoides* exhibited significant synergistic effect while antagonism was observed mostly among compounds from *C. imberbe*. This study indicates that there may be synergistic as well as antagonistic effects in naturally isolated compounds. Because there was insufficient material to evaluate the statistical significance of the results, the conclusion should be considered tentative.

Compounds **1** and **3** exhibited very good anti-inflammatory effect against 3α-hydroxylsteroid dehydrogenase enzyme with IC_{50} of 0.30 µg/ml. Compounds **4, 5** and **8** indicated a mild inhibitory effect.

Compound **3** had a moderate cytotoxic effect of CC_{50} = 10, 5 µg/ml against Hela and the rest of the compounds **1, 2, 4, 5, 6,** and **7** were generally non-toxic. Compound **3** showed a strong antiproliferative activity against both L-929 and K-562 cells with GI_{50} of 9 µg/ml, and 8.5 µg/ml, and moderate antiproliferative activities of 16.5 µg/ml, 13, 5 µg/ml for compound **5** against both cell lines respectively.

CHAPTER 8

GENERAL CONCLUSION

8.1 Introduction

The increasing use of antibiotics and misuse by over prescribing and or poor patient compliance has led to the development of bacteria resistant to antibiotics. Medicinal plant research offers a good chance of discovering new prototype drugs (Malone, 1983). According to ethnobotanical literature the genus *Combretum* is used widely for a variety of conditions in African traditional medicine. Members of this genus have the following biological activities: antibacterial, antifungal, anti-inflammatory, diuretic, and molluscidal (Hutching *et al.* 1996). Some members of Combretaceae have been found to have compounds with antibacterial activity. Eloff (1999a) has previously reported that *Combretum imberbe* and *Combretum padoides* extracts had antibacterial activity. Therefore, the aim of this work was to isolate, chemically and biologically characterize antibacterial compounds present in *Combretum* section Hypocrateropsis in a bioassay guided process, with the objectives of:

- ✓ Selecting the most active plant specie(s)
- ✓ Selecting and evaluating the best fractionation procedure for isolation
- ✓ Isolating and determining the chemical structure of antibacterial compounds
- ✓ Determining the biological activities of isolated compounds
- ✓ Determining the effect of synergism on combinations of isolated compounds
- ✓ Evaluate how well phytochemistry agrees with taxonomy based on morphology.

The degree to which the above aim and objectives have been met is briefly outlined in the following sections:

8.2 Evaluation on the best preliminary fractionation procedure

In an attempt to isolate and characterize the antibacterial compounds in *C. imberbe* and *C. padoides,* two fractionation procedures were evaluated as preliminary processes. Initially, dried ground plant materials were extracted directly with acetone for screening purpose and bulk material was extracted serially and exhaustively with solvents of increasing polarity (hexane, DCM, acetone, methanol) for isolation purposes. DCM was the best extractant of antibacterial compounds according to bioautography and MIC analysis. The DCM extract was therefore used for the isolation of antibacterial compounds through a bioassay-guided

approach. The solvent-solvent fractionation method and vacuum liquid chromatography were used as preliminary fractionation procedures in order to evaluate the best approach toward quick and easy isolation of antibacterial compounds. The solvent-solvent fractionation was a better preliminary process because more antibacterial compounds were isolated through this approach compared to the vacuum liquid chromatgraphy approach.

8.3 Isolation and chemical characterization of antibacterial compounds

Screening of the 4 plant species of the Hypocrateropsis section was done and species for further work were selected on the basis of number of antibacterial compounds as well the MIC of extracts. *Combretum imberbe (*8 antibacterial compounds, MIC 0.60 mg/ml against *S. aureus)* and *C. padoides* (7 antibacterial compounds, MIC 0.60 mg/ml against *S. aureus*) were selected for further work. Both screening and isolation processes were monitored by the TLC analysis using three different solvent systems developed in our laboratory. BEA separated non- polar compounds, CEF separated compounds of intermediate polarity and EMW separated polar compounds. EMW and CEF were the best mobile phases for separation of the active compounds. BEA was not a good system for TLC separation because most of the active compounds were of intermediate polarity. Vanillin sulphuric acid and *p*-anisaldehyde-sulphuric acid dectection reagents were initially used. However, more compounds were visible with vanillin sulphuric acid than with the anisaldehyde spray reagent. Vanillin sulphuric acid spray reagent was therefore routinely used; it appears to be a good spray reagent for the detection of triterpenes. Vanillin-sulphuric acid spray reagent did not reveal many of the compounds that were seen under UV light.

Column chromatography using Silica gel 60 and Sephadex LH 20 was the main approach used for isolating compounds. Eight antibacterial compounds were isolated through the above process. NMR, MS and IR analysis were used to determine the chemical structures of the compounds. Five compounds were isolated from *C. imberbe* and elucidated as: 1,3-dihydroxyl-12-oleanen-29- oic acid (**1**), 3-hydroxyl-12-oleanen-29-oic acid (**2**), 3, 30-dihydroxyl-12-oleanen-22-one (3), 1,3,22-trihydroxyl-12-oleanen-29-oic acid (**4**) and 1α, 23 β -dihydroxyl-12-oleanen-29-oic acid-23β-O-α-2,4-diacetylramnopyranoside (**5**) All of them were triterpenes with the olean-12-ene skeleton. Compound **5** was a triterpene glycoside, which has not been reported before. Three compounds were isolated from *C. padoides* and also elucidated as: {1α, 23β -dihydroxyl-12-oleanen-29-oic acid-3β-O-α-4-acetylramnoprranoside (**6**), 1, 22-dihydroxyl-12-oleanen-29-oic acid **(7**), and 24-ethylcholesta-7, 22, 25-trien-3-ol-O-β-D-glucopyranoside (**8**). Two 1α, 23β -dihydroxyl-12-oleanen-29-oic acid-3β-O-α-4-acetylramnoprranoside (**6**) 1, 22-dihydroxyl-12-oleanen-29-oic acid **(7**)) had the olean-12-ene skeleton and the other 24-ethylcholesta-7, 22; 25-trien-3-ol-O-β-D-glucopyranoside (**8)**

was a steroid glycoside. Compound **6** from *C. padoides* was a new triterpene glycoside. Generally, the results of the study expand our knowledge on the phytochemistry of Combretaceae. It is evident that the above new structures will join the global natural product database and may serve as models for the synthesis of new antibacterial compounds.

8.4 Biological characterization of plant species and isolated compounds

In vitro anti-infective, anti-inflammatory, anti-proliferactive and cytotoxic activities were tested for most of the compounds depending on the quantity of material that was available. Some of the compounds had a broad-spectrum antibacterial activity against both Gram-positive (*S. aureus* and *E. faecalis)* and Gram-negative organisms (*E. coli* and *P. aeruginosa*). The MIC of active compounds against the Gram-positive organisms ranged from 0.030 to > 0.25 mg/ml and 0.016 to > 0.25 mg/ml for the Gram-negative strains .The agar well diffusion method was carried out to determine the sensitivity of more bacteria and fungi strains against the compounds. Most of the compounds showed zones of inhibition ranging from 0 to 26 mm with Compound **1** showing the highest activity (26 mm) against *Mycobacterium vaccae.* Compound **6** was active against two fungi; *Sporobolomyces salmonicolor* (18 mm) and *Candida albicans* (26 mm). Limited data has been reported on the anti-microbial activity of components isolated from Combretaceae. This study confirms the first report of Katerere *et al*., (2002) on the antibacterial activity of triterpenes isolated from African Combretaceae and also gives credence to the indigenous use of *C. imberbe* in tradition medicine against bacteria related diseases.

The anti-inflammatory activity of the compounds were tested against 3α-hydroxylsteroid dehydrogenase enzyme (Penning, 1983) and compounds **1** and **3** had good anti-inflammatory activity with IC_{50} value of 0.30 µg/ml each hence confirmed the anti-inflammatory activity of some *Combretum* species (Hutchings *et al*., 1996).

The antiproliferative and cytotoxic efficacy of compounds **1-8** were tested *in vitro* against (L-929, k-562) and (Hela) cell lines respectively. Compounds **3** and **5** showed a strong antiproliferative activity against both L-929 and K-562 cell with GI_{50} of 9 µg/ml, 8, 5 µg/ml for compound **3** and moderate antiproliferative activities of 16.5 µg/ml, 13, 5 µg/ml for compound **5**. Compound **3** showed a moderate cytotoxic effect of CC_{50} = 10, 5 µg/ml against Hela

Based on the MIC values of the new compounds (**5** and **6**) and compound **1** against *S. aureus and E. coli* further investigation into the biological activity of these compounds or derivatives against more resistant

strains may be fruitful. Generally, *C. imberbe* and *C. padoides* leaves contain many antibacterial compounds of which only few were isolated and characterized. Because this plant contains many compounds, further work may be carried out on isolation and characterization of other antibacterial compounds present in this species. These results validate the ethnobotanical use of many *C.* species for bacteria infections. In future work more antibacterial compounds that are present in low concentrations could be isolated by statrting with large quantity of plant material.

8.5 Evaluation of how well phytochemistry agrees with taxonomy based on morphology

All compounds isolated from the two plant species are pentacyclic triterpenes. Classification based on morphology placed *C. imberbe* and *C. padoides* in the section Hypocrateropsis. TLC (presence of terpenes by vanillin sulphuric acid spray reagent) analysis has shown that members of the Hypocrateropsis section have similar chemical profile. The isolation result substantiates a biogenetic link between members of the Hypocrateropsis.The seven antibacterial flavanoids isolated from *C. erythrophyllum* a member of the section Angustimarginata (Martini *et el.,* 2004b). The main antibacterial compound isolated from *C. woodii* also a member of the Angustnarginata was a bibenzyl.

The chemical composition of antibacterial compounds appears to support the current taxonomical classification of the sections in the *Combretum* genus. Related studies have shown the presence of triterpenes in related generals of Combretaceae. The result of this work might indicate a chemotaxonomical correlation between generals of combretaceaes.