CHAPTER NINE

Isolation and characterization of antimicrobial and antioxidant compounds from *Bauhinia galpinii* and *Combretum vendae*

9.0. Introduction

One of the cardinal objectives in medicinal plant research and development is identification, isolation and characterization of the bioactive components present in an extract. Medicinal plant extracts are inherently complex mixture of diverse chemical components. Separation of the active components of plant phytochemicals from the inactive components are categorized into three parts: extraction, purification and chromatography. Extraction and purification involved sample preparations schematically represented in Fig. 9.1 and Fig. 9.2. Various chromatographic methods are available for qualitative and quantitative (TLC fingerprint, high performance liquid chromatography (HPLC) fingerprints) as well as for isolations (open column chromatography (OCC), vacuum liquid chromatography (VLC), HPLC, high-speed counter-current chromatography (HSCCC), gas-liquid chromatography (GLC) and/or gel permeation chromatography (GPC)). The principles of separation are based on molecular size, adsorption to the stationary phase, polarity and solubility in the mobile phase.

Structural information on isolated compounds are usually obtained from different spectroscopic techniques namely: nuclear magnetic resonance spectroscopy (NMR), mass spectroscopy (MS), and to a lesser extent infrared spectroscopy (IR) and ultraviolet-visible spectroscopy (UV-visible).The characteristic features of each NMR experiments are summarized in Table 9.1.

Table 9.1 NMR experiments commonly applied for natural product structural elucidation (Simpson *et al.*, 2011)

NMR experiment	**Information/interpretation**
Proton NMR (1D ^{1}H NMR)	Quantitative overview of the distribution of protons in a sample.
Carbon-13 NMR (1D ^{13}C NMR)	Can provide a quantitative overview of the carbon distribution.
Distortionless enhancement through polarization transfer (DEPT) (1D ^{13}C NMR)	Separate the carbon of a compound into primary (CH_3), secondary (CH_2), Tertiary (CH) and quaternary (C) spectra.
^{1}H-^{1}H Correlation spectroscopy (COSY) (2D ^{1}H NMR)	Connectivity information of protons on adjacent carbons. Cross-peaks connect the chemical shifts of protons that are coupled. Symmetrical cross peaks appear around a central diagonal.
^{1}H-^{13}C Heteronuclear single quantum correlation (HSQC) and heteronuclear multiple quantum correlation (HMQC) (2D ^{1}H- ^{13}C NMR)	^{1}H–^{13}C 1 bond correlation. Cross peaks represent carbon chemical shifts in one dimension and proton chemical shifts in the other dimension.
^{1}H-^{13}C Heteronuclear bond multiple correlation (HMBC) (2D ^{1}H- ^{13}C NMR)	^{1}H–^{13}C 2–4 bond correlations. Quaternary carbons are observed. Connectivity information is read as vertical lines.

9.1.1. Column chromatography

Isolation of such compounds is usually carried out by open column chromatography under gravitation force using silica gel, sephadex, polyamides or reverse phase (RP) mode on C8 or C18-bonded silica gel stationary phase. The separation of individual compounds from the complex extract mixture is based on the compound characteristic ability for the stationary phase in the column relative to the polarity of the mobile phase. Changing the polarity (gradient elution) of the mobile phase will allowed all target compounds to elute in a sequential manner. The chromatographic process should be rapid, do not lead to decomposition of compounds, material loss, or formation of artefacts.

Open column chromatography is simple, cheap and universally practiced despite some obvious disadvantage of method being slow and often produces irreversible adsorption of sample onto the stationary phase. The method is also encumbered with large sample and solvent requirement. The bioactive compounds of interests in this project are non-volatile.

9.1.2. Mass spectrometry

Mass spectrometry (MS) is an important physico-chemical tool applied for structural elucidation of compounds from natural products including medicinal plants. The fundamental principle of MS is the use of different physical means for sample ionization and separation of the ions generated based on their mass (m) to charge (z) ratio (m/z) (Rijke *et al.*, 2006). The ionization techniques available include electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI), electron ionization (EI), chemical ionization (CI), fast atom bombardment (FAB), and matrix-assisted laser desorption ionization (MALDI) (Rijke *et al.*, 2006). Mass spectrometry has high sensitivity with detection limit of fentogram compared to NMR with sensitivity limit of nanogram range and above (Simpson *et al.*, 2011). The high sensitivity and the flexibility for hyphenation with other chromatographic technique made MS a versatile analytical instrument.

9.2. Materials and Methods

9.2.1. Preparation of plant extracts

The extraction and fractionation protocol was followed as described in Chapter 3. The schematic diagrams of the extraction, fractionation and isolation processes for *Combretum vendae* and *Bauhinia galpinii* are presented in Fig 9.1 and 9.2.

9.2.2. Bioautography

The bioautography against bacteria (*E. coli and S. aureus*) protocol were followed as described in chapter 4 while the TLC-DPPH antioxidant assay were carried out as described in chapter 5.

9.2.3. Isolation of bioactive terpenoids from *Combretum vendae*

The n-Hexane and ethyl acetate fractions showed one and two clear zone(s) of microbial growth inhibition respectively. The two fractions were subjected to gravitational column chromatography on silica gel (2.5cm×73cm using 150 g silica, particle size 0.063–0.200 nm, Merck 70–230 mesh ASTM) using the solvent mixture of hexane: ethyl acetate starting with 100% n-Hexane, 99: 1, 98:2, 97:3, 96:4, 95:5, 94:6, 93:7 and finally 90:10 as mobile phase. Schematic representation of the isolation procedure is presented in Fig 9.1.

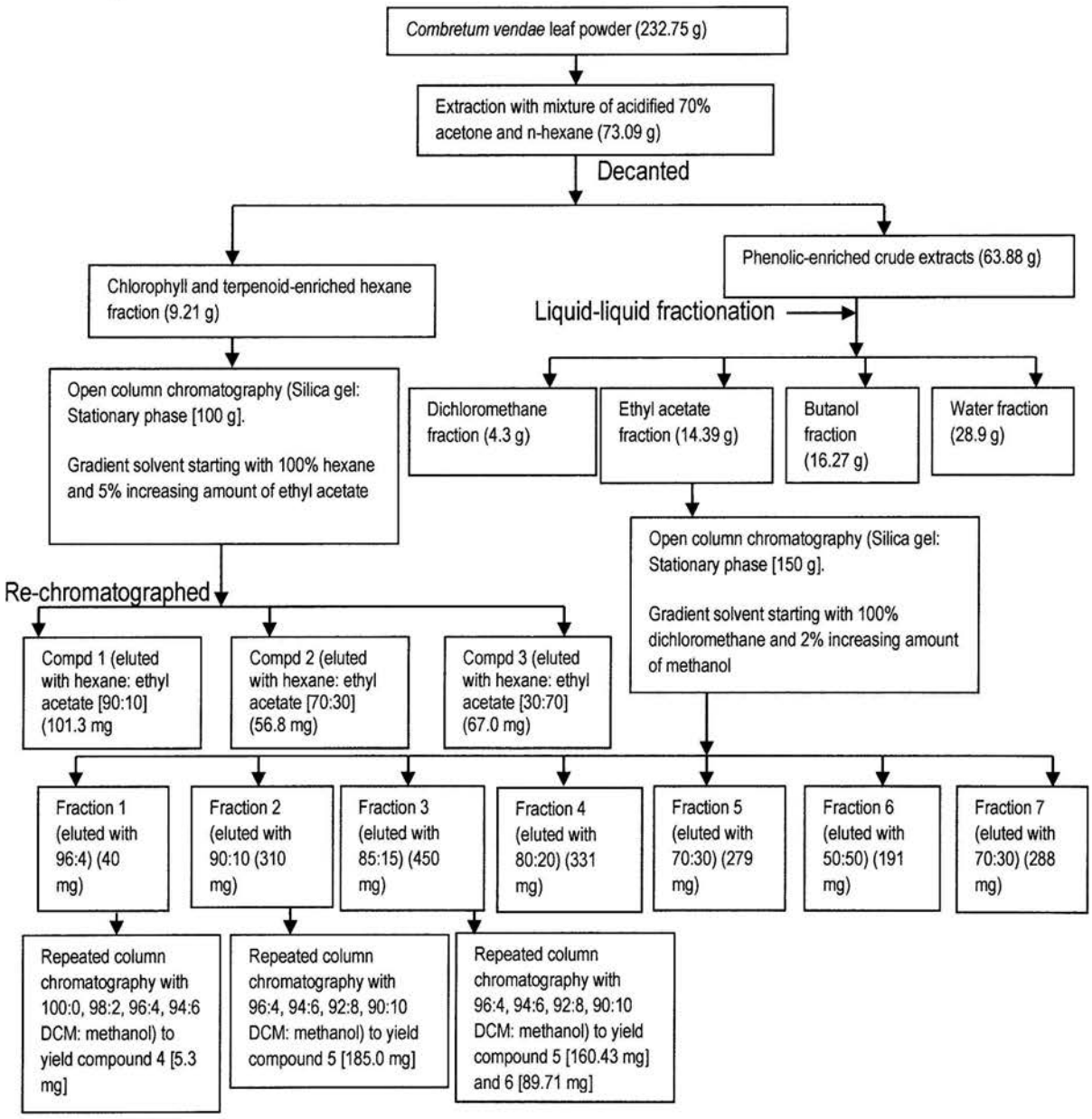


Fig. 9.1. Extraction, fractionation and isolation of bioactive compounds from the leaf extract of *Combretum vendae*

The dried fractions were reconstituted (10 mg/ml) and 10 µl of the aliquot spotted on TLC. Three mixtures of n-Hexane: ethyl acetate (90:10; 95:5 and 98:2) was used to develop the plates. The fractions with R_f corresponding to the R_f values of bioautography assay were combined. The purification of the compounds was achieved by repeated column chromatography until single spot was obtained for each compound using three different mobile phases to develop the TLC.

9.2 4. Isolation of bioactive phenolics from *Combretum vendae*

The ethyl acetate, n-Butanol and residual water fractions exhibited good antimicrobial and antioxidant activities. In TLC-DPPH assay four clear zones of antioxidant components were observed in ethyl acetate fraction. The fraction was subjected to open column chromatography under gravitational force (2.5cm×73cm using 150 g silica, particle size 0.063–0.200 nm, Merck 70–230 mesh ASTM) using the solvent mixture of dichloromethane: methanol starting with 100% dichloromethane, 98:2, 96:4, 94:6, 92:8, 90:10, 85:15, 80:20, 70:30, 60:40, 50:50 as mobile phase. The eluents were monitored with DPPH-TLC antioxidant assay and vanillin/H_2SO_4 spray reagent. The fractions with the same compounds were combined and subjected to further cleaning by re-chromatography until single spots were obtained on TLC chromatogram using three different mobile phases. Schematic representation of the isolation procedure is presented in Fig 9.1.

9.3. Isolation of compounds from *B. galpinii*

9.3.1. Isolation of bioactive terpenoid from *Bauhinia galpinii*

The n-Hexane fractions showed one clear zone of microbial growth inhibition and was subjected to open column chromatography under gravity on silica gel (2.5cm×73cm using 150 g silica, particle size 0.063–0.200 nm, Merck 70–230 mesh ASTM) using the solvent mixture of hexane: ethyl acetate starting with 100% n-Hexane, 98:2, 96:4, 94:6, 92:8 and finally 90:10 as mobile phase. The eluent were monitored using TLC and vanillin/H_2SO_4 spray. The fractions containing the target compound was combined and the chromatography process was repeated until a single spot using three mixtures of n-Hexane: ethyl acetate (90:10; 95:5 and 98:2) as mobile phases for TLC chromatogram was obtained. Schematic representation of the isolation procedure is presented in Fig 9.2.

9.3.2. Isolation of bioactive phenolics from *Bauhinia galpinii*

The ethyl acetate, n-Butanol and residual water fractions exhibited good antimicrobial and antioxidant activities. In TLC-DPPH assay four clear zones of antioxidant components were observed in ethyl acetate and butanol fractions. The fractions were subjected to open column chromatography under gravitational force (2.5cm×73cm using 150 g silica, particle size 0.063–0.200 nm, Merck 70–230 mesh ASTM) using the acidified solvent mixture of dichloromethane: methanol starting with 100% dichloromethane, 98:2, 96:4, 94:6, 92:8, 90:10, 85:15, 80:20,

70:30, 60:40, 50:50 as mobile phase. The eluents were monitored with DPPH-TLC antioxidant assay and vanillin/H_2SO_4 spray reagent. The fractions with the same compounds were combined and subjected to further cleaning by re-chromatography using silica gel or Sephadex L20 until single spots were obtained on TLC chromatogram using three different mobile phases. Schematic representation of the isolation procedure is presented in Fig 9.2.

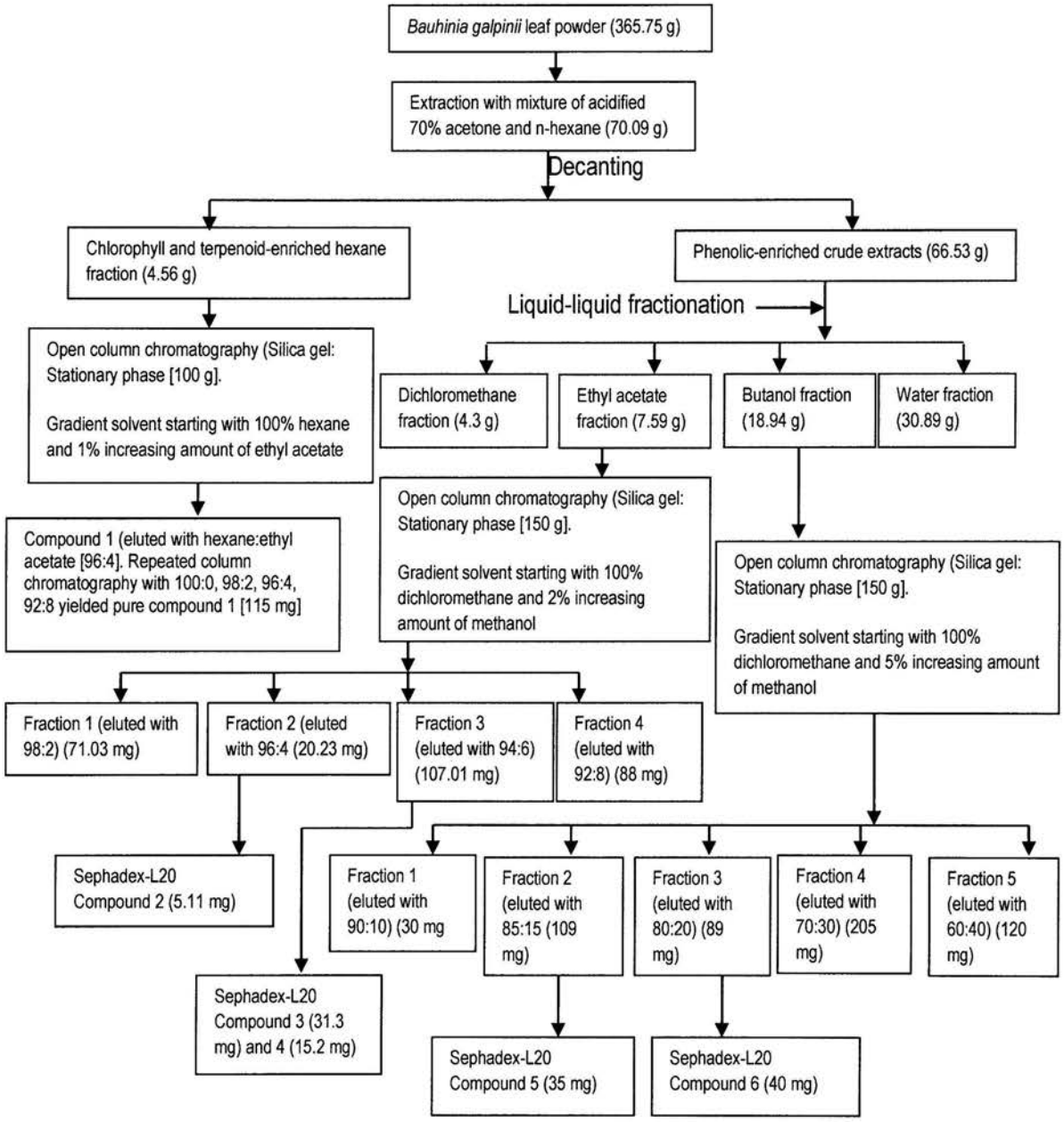


Fig. 9.2. Extraction, fractionation and isolation of bioactive compounds from the leaf extract of *Bauhinia galpinii*

9.4 Characterization of the isolated compounds

9.4.1 NMR spectroscopy

One dimensional (1D) (^{1}H and ^{13}C) and two dimensional (2D) NMR spectra (^{1}H-^{1}H COSY, HMQC and HMBC) NMR spectra were recorded on a Varian-NMR-vnmrs 600 spectrometer with tetramethylsilane (TSM) as internal standard. Standard pulse sequences were used for homo- and heteronuclear correlation experiments. ^{1}H NMR spectra were measured at 599 MHz whereas ^{13}C NMR spectra were run at 150 MHz. Multiplicities of ^{13}C NMR resonances were determined by DEPT experiments. All NMR experiments were performed at constant temperature (27 °C) using software supplied by the manufacturer, employing deuteriochloroform, deuteriomethanol, or deuteriodimethylsulphoxide as solvent on the basis of solubility of the sample and literature data.

9.4.2 Mass Spectroscopy:

Electrospray ionization mass spectrometric analyses (negative and positive mode) were carried out to obtain the molecular weight and fragmentations patterns of the isolated compound(s) using TOF mass spectrometer (WATERS HPLC).

9.4.3 UV spectroscopy:

The UV-spectrum of the isolated bioactive compound(s) was recorded using Agilent 1200 UV-Visible spectrophotometer.

9.5 Results

9.5.1 Identification of the chemical structures of isolated compounds from *Combretum vendae*

The n-Hexane and ethyl acetate fractions from the acidified 70% acetone leaf extract of *Combretum vendae* A. E. van Wyk through bioassay guided fractionation were repeatedly subjected to gravity column chromatography to yield one pure, mixture of four position isomer antimicrobial triterpenoids and two stilbene glucopyranoside. The structures of the compounds were determined by extensive NMR techniques and chemical methods mainly by 1D NMR (^{1}H, ^{13}C and DEPT) and 2D NMR (HSQC, HMBC and COSY), ESIMS, UV-visible spectra and by comparison with the literature data. The chemical structures of the compounds compounds isolated from *Combretum vendae* are presented in Fig 9.3.

Compound 1 was obtained as an amorphous white powder. Detailed analyses of the 1D and 2D NMR spectra indicated the presence of 30 carbons which revealed 7 methyl, 9 methylene, 6 methane, 6 quaternary, 1 carboxylic acid at δ_C 178.9, an olefinic broad triplet proton at δ_H 5.25 (H-12) coupled to a carbon at δ_C 125.4, a quaternary carbon at δ 138.8.0 (C-13) and a β-18 proton at δ_H 2.05 characteristic of signal of an ursol-12-en

skeleton (Appendix 9.1). The Comparison of these NMR data with the literature confirms the compound as ursol-12-en-28-oic acid (ursolic acid) (Mahato and Kundu, 1994).

Compound 2 and 3 were obtained as position isomeric mixture; the TLC fingerprint indicated unresolved single spot with three different mobile phases. However, the 1D and 2D NMR spectra exhibited chemical shift characteristic of both olean-12-ene and ursol-12-ene. ^{13}C NMR (DMSO): δ 179.7(C-28, olean), 178.0 (C-28, urs), 145.08 (C-13, olean), 122.72 (C-12, olean), 139.42 (C-13, urs), 125.55 (C-12, urs), 41 (C-18, olean), 53 (C-18, urs), 27.97 (C-19, olean), 39.07 (C-19, urs) and 46.13 (C-20, olean), 39.02 (C-20, urs) (Appendix 9.2). Comparing the data with the literature values, the two compounds in the mixture were identified to be corosolic acid and maslinic acid (Mahato and Kundu, 1994)

Compound 4 and 5 were also obtained as position isomeric mixture. The spectra 1D and 2D were similar to the spectra of compound 2 and 3 except the presence of additional hydroxyl at C-24) (appendix 9.3). From the correlation data and literature values, compound 4 and 5 were identified to be asiaitic acid and arjunolic acid respectively (Mahato and Kundu, 1994).

Compound 6 was obtained as an amorphous powder from the ethyl acetate fraction. The proton NMR spectrum shows signal for apigenin: δ_H 6.19 (d, J=2.0 Hz, H-6), 6.38 (d, J=2.0 Hz, H-8), 7.2 (d, J=2.1 Hz, H-3), 6.50 (d, J=8.9 Hz, H-3' and H-5'), and 7.30 (d, J=2.1 Hz, 8.9, 2H' and H-6'). The ^1H NMR spectral data correspond to the literature values (Zhang *et al.*, 2011).

Compound 7 was obtained as creamy glass-like solid mass and deduced to have molecular formula $C_{23}H_{29}O_{11}$ with molecular weight 481 by EIMS. The ^{13}C-NMR spectrum indicated 17 carbon signals due to aglycone and six carbon signals of a glycoside group. The signal consist twelve aromatic signals, three methoxy and two aliphatic (Appendix 5). The ^1H-NMR spectra revealed the presence of two aromatic benzylic rings. The ^1H detected heteronuclear multiple-bond connectivity (HMBC) spectrum indicated long-range correlations from ^1H-1a'(δ_H 2.9, 3.0) to C-1' (δ_C 128.55), C-2' (δ_C 144.35), C-6' (δ_C 118.95), C-1a (δ_C 36.96) and C-1 (δ_C 132.72). Long range correlation observed between ^1H-1a (δ_H 2.7) and C-1 (δ_C 132.72, C-2 (δ_C 106.16), C-6 (δ_C 106.16), C-1a' (δ_C 31.73) and C-1' (δ_C 128.55). Additional long-range correlation were observed between ^1H-2, 6 (δ_H 6.5) and C-1 (δ_C 132.72), C-3, C-5 (δ_C 148.16), C-4 (δ_C 133.79), C-1a (δ_C 36.96); ^1H-5' (δ_H 6.7) to C-1' (δ_C 128.55), C-3' (δ_C 139.71), C-6' (δ_C 118.95), C-4' (δ_C 147.26) and ^1H-6' (δ_H 6.6) to C-1a' (δ_C 31.73), C-3' (δ_C 139.71), C-4' (δ_C 147.26). Long-range correlation also observed between methoxyl signal (δ_H 3.7) and the C-3, C-5 (δ_C 148.16), C-4' (δ_C 147.26) (appendix 9.4). Based on these data, the aglycone of compound 7 was determined to be stilbenes derivative. The signals for anomeric proton and carbon (δ_H 4.5 and δ_C 106.26) indicated the presence of a sugar moiet. The long-range correlation in the HMBC experiment between the anomeric proton signal (δ_H 4.5) of the β-D-glucopyranosyl group and the C-2' signal (δ_C 144.35) confirmed the position of the attachment of the

glucopyranosyl moiety on the phenolic ring. The structure of compound 6 was determined to be combretastatin B5-O-2'-β-D-glucopyranoside. The EIMS, 1D (^{1}H and ^{13}C, DEPT), and 2D (HSQC, HMBC, COSY) data correlate with literature information on the compound (Pettit *et al.*, 1985).

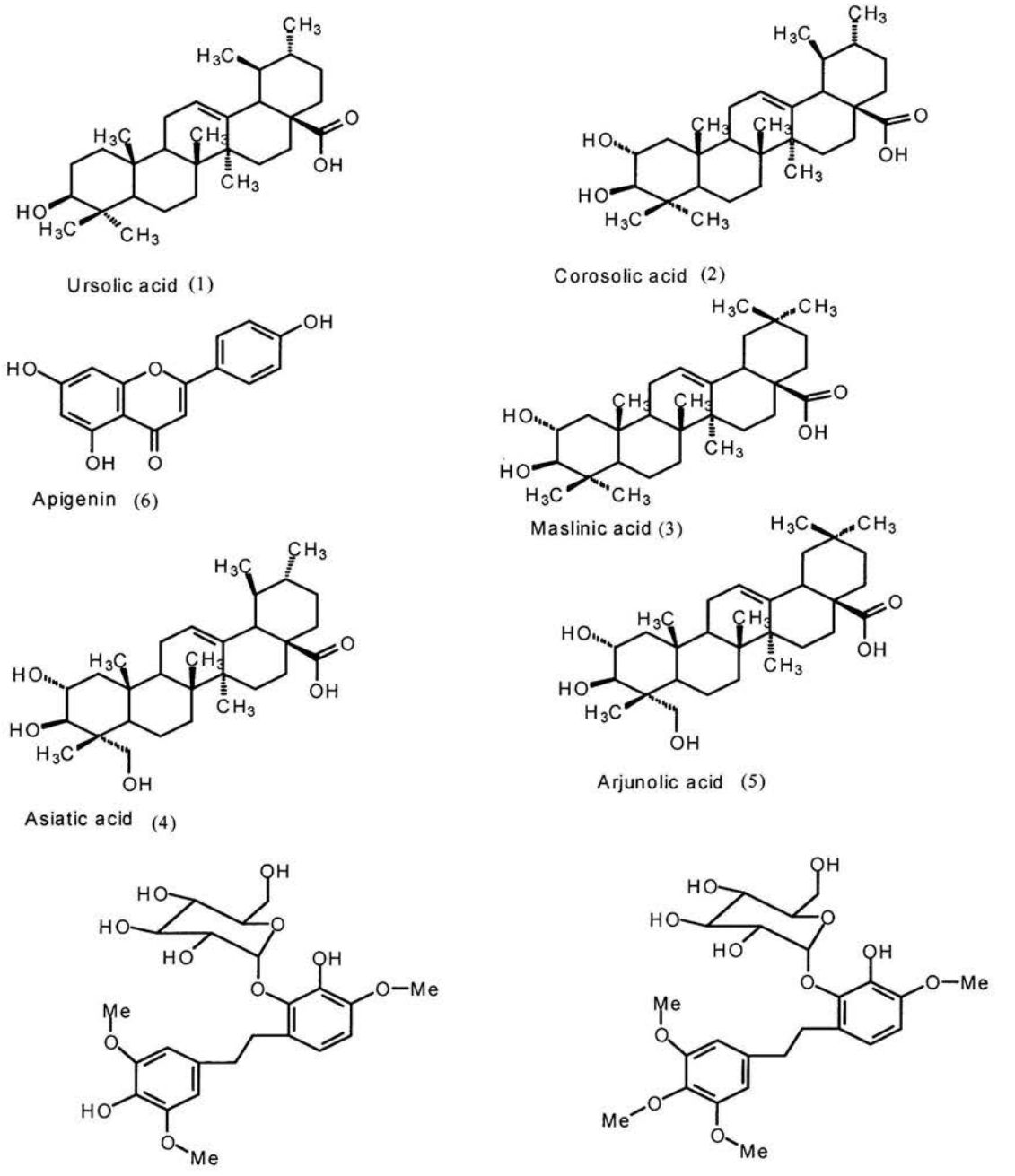


Fig 9.3 Chemical structures of isolated bioactive compounds from 70% acetone leaf extract of *Combretum vendae*

Compound 8 had molecular weight of 496 and the molecular formula was deduced to be $C_{24}H_{32}O_{11}$ by EIMS. The compound differs from compound 6 by the presence of extra methyl group. This was confirmed by 1D and 2D NMR spectra. From HSQC one additional methoxyl signal (δ_H 3.6 and δ_C 59.69) having long range correlation C-4 (δ_C 135) (appendix 9.5). The long range correlation from HMBC experiment between ^{1}H-4 proton of the glucopyranosyl moiety (δ_H 3.7) and the C-2' signal (δ_C 143.78) confirmed the position of the structure of compound 7 to be combretastatin B1-O-2'-β-D-glucopyranoside. The EIMS, 1D (^{1}H and ^{13}C, DEPT), and 2D (HSQC, HMBC, COSY) data correlate with literature information on the compound (Schwikkard *et al.*, 2000).

9.5.2 Antimicrobial assay of isolated compound from *C. vendae*

Antimicrobial activities of the isolated compounds against standard and clinical isolate pathogens expressed as MIC are presented in Table 9.1. Some of the compounds exhibited good microbial growth inhibitory potential worthy of pharmacological considerations with MIC ranging from 3.9-31 µg/ml.

Table 9.2: Minimum inhibitory concentration (µg/ml) of the isolated compounds from the leaf extract of *Combretum vendae*

Microorganisms	Compound 1	Compounds 2 and 3	Compounds 4 and 5	Compound 7	Compound 8
E. coli	62	250	250	250	250
E. faecalis	31	31	31	31	31
S. aureus	62	125	125	125	62
P. aeruginosa	125	125	62	250	250
C. albicans (M0825)	15	7.8	7.8	31	31
C. albicans (M0824)	62	62	31	125	125
C. albicans (1051604)	3.9	3.9	3.9	3.9	3.9
C. albicans (1051608)	7.8	7.8	7.8	15	15
C. albicans (ATCC 10231)	7.8	15	7.8	31	31
C. neoformans	15	15	15	125	125
A. fumigatus	31	31	31	250	250

1: Ursolic acid, 2 and 3: mixture of maslinic and corosolic acid, 4 and 5: mixture of asiatic and arjulonic acid, 6: combretastatin B5-O-2'-β-D-glucopyranoside.7: combretastatin B1-O-2'-β-D-glucopyranoside. *C. albicans* (M0824), *C. albicans* (M0825), *C. albicans* (1051604), *C. albicans* (1051608) are clinical isolate obtained from National Health Laboratory Service, Pretotia, South Africa.

9.5.3 Identification of the isolated bioactive compounds from *Bauhinia galpinii*

The combine TLC fingerprint, TLC-DPPH assay and bioactivity guided fractionation of acidified 70% acetone leaf extract of *B. galpinii* detected four major flavonoids from ethyl acetate fraction with antioxidant activity. Bioautography against fungal and bacterial pathogens revealed two microbial inhibitory growth spot in Hexane

fraction and one in DCM fraction respectively. The bioactive compounds were isolated from each fraction using open column chromatography with silica gel as stationary phase. The phenolics compounds were further purified using Sephadex L-20 as stationary phase and acetone/methanol (50:50) as mobile phase at a rate of 2ml/5min. The chemical structures of the compounds were determined by detailed nuclear magnetic resonance (NMR) techniques including the one dimensional (1D) NMR (proton (^{1}H), carbon-13 (^{13}C) and distortion enhancement DEPT) and two dimensional (2D) NMR (HSQC, HMBC and COSY). Mass spectrometry and the fragmentation patterns of the compounds were extensively used for the structural elucidation.

Compound 9 obtained as white amorphous powder from hexane fraction was characterized by ^{1}H NMR spectra (in $CDCl_3$), ^{13}C-NMR spectra (in $CDCl_3$), HSQC, HMBC, DEPT and COSY. The ^{1}H NMR spectrum of 9 showed a one-proton doublet at δ_H 5.33 (J¼5.5 Hz) assigned to a vinylic H-6 proton. A one-proton broad multiplet at δ_H 3.46 with half-width of 18.5 Hz was attributed to carbinol H-3 proton. Two three-proton broad signals at δ_H 0.67 and 1.01 were attributed to a tertiary C-18 and C-19 methyl protons. A six-proton broad signal at δ_H 0.83 was associated with C-29 and C-20 methyl protons. Two three proton doublets at δ_H 0.91 (J¼6.2 Hz) and 0.85 (J¼6.3 Hz) were due to secondary C-26 and C-27 methyl protons. The remaining methylene and methine protons appeared between δ_H 2.50 and 1.27. The presence of all the methyl signals in the range δ_H 1.01–0.67 suggested that all these functionalities were located on the saturated carbons. The ^{13}C NMR spectrum of 9 exhibited signals for vinylic carbons at δ_C 140.97 (C-5) and 121.96 (C-6). The two carbinol signals appeared at δ_C 72.04 (C-3) and 76.86 (CH_3 $\underline{CH_2}O$-) respectively. The carbon signals in the upfield region at δ_C 12.20, 21.30, 19.25, 20.04, 19.62, 12.08 and 19.00 were associated with the methyl functionalities. The remaining methylene and methine carbon resonated between δ_C 56.99 and 23.29. The ^{1}H–^{1}H COSY spectrum of 9 showed correlations of H-6 with H2-7 and H-8; H-3 with H2-2, H2-4 (appendix 9.6). The ^{1}H and ^{13}C NMR spectral data of steroidal nucleus of 9 were compared with related steroidal constituents (Alam *et al.*, 1994). On the basis of the foregoing discussion, the structure of 9 was elucidated as 3β-ethyl stigmast-5-en-ol (3β-ethyl sitosterol). This compound may be an artefact of sitosterol.

Compound 10 and 11 was isolated from the ethyl acetate fraction as a yellow powder by repeated gravitational column chromatography on silica gel and Sephadex L-20 stationary phases. The ^{1}H NMR spectrum in deuterated methanol of 10 showed signals for quercetin: δ_H 6.19 (d, J=2.0 Hz, H-6), 6.38 (d, J=2.0 Hz, H-8), 7.57 (d, J=2.1 Hz, H-2', δ_C 116.30), 6.86 (d, J=8.9 Hz, H-5', δ_C 116.78), and 7.56 (d, J=2.1 Hz, 8.9, H-6', δ_C 121.65). Carbon 13 NMR (100 MHz, in ppm, methanol-d4) shows 15 signals and the data (appendix 6) correlated well with literature for 3, 5, 7, 3', 4'-pentahydroxyflavone (Quercetin) (Said *et al.*, 2009)

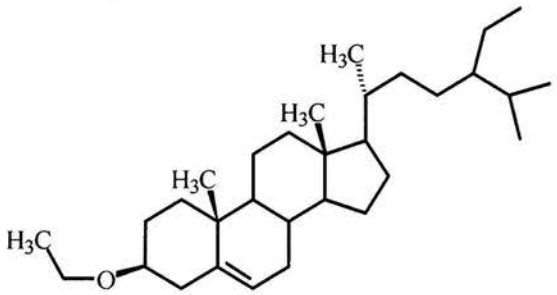
The ^{1}H NMR of compound 11 in deuterated methanol at 599.74MHz revealed the presence of myricetin aglycone: δ_H 7.383 (s, H-2',6', δ_C 107.09) for the B phenyl ring, and 6.369 (d, H-8, δ_C 92.95), 6.338 (d, H-8, δ_C 92.95), 6.172 (d, H-6, δ_C 97.80) and 6.170 (d, H-6, δ_C 97.80) for the meta substituted A phenyl ring. The structure

was confirmed by ^{13}C-NMR which showed 15 signals without methoxy or glycoside substituent and 2D correlation (HSQC and HMBC) (appendix 9.7). The data correlated well with literature data for 3, 5, 7, 3', 4', 5'-hexahydroxyflavone (myricetin) (Said *et al.*, 2009)

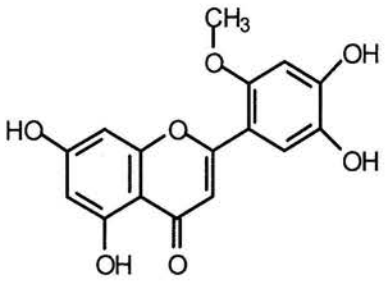
The NMR spectrum of compound 12 was typical for a flavone with one meta-substituted and para-substituted phenolic moiety. The UV spectrum showed λ_{max} 261.27 and 369.27 nm which compared favourably with 263 and 367 nm reported by Abdurrahman and Moon, 2007 for isoetin moiety. The compound was assigned molecular formula $C_{16}H_{10}O_7$ with the aid of a peak observed in the ESI-MS experiment at 315.054. The carbon chemical shifts were assigned by the combination^{1}H-^{13}C HSQC and long range couplings in the ^{1}H-^{13}C HMBC experiments. The ^{1}H-NMR spectrum was indicative of five aromatic protons, one methoxyl group. The ^{1}H and ^{13}C NMR spectra data correlated with those of isoetin flavone moiety (Abdurrahman and Moon, 2007; Pauli and Junior, 1995; Voirin *et al.*, 1975) (Table 1). The ^{1}H-^{13}C HSQC correlations were used to assign signal at δ_H 7.19, 6.27 and 6.40 to the protons at C-3 (s, δ_C 108.85), C-6 (d, J = δ_C 99.97) and C-8 (d, J = δ_C 94.86) positions of A ring, and the signal at δ_H 6.65 and 7.38 to the proton at C-3' (s, δ_C 101.36) and C-6' (s, δ_C 114.45) positions of B ring of the isoetin moiety respectively. The long range coupling in the HMBC presented in appendix 9.10 also supported the isoetin flavone moiety. The methoxy protons signal at δ_H 3.8 was correlated with the quaternary carbons (C-2') at δ_C 153.28 indicating the attachment of methoxy group at the carbon (appendix 9.8). From all the correlations, compound 13 was determined to be a new flavone named as 5, 7, 4' 5' tetrahydroxy-2'-methoxyflavone (isoetin 2'-methyl ether) or 5, 7, 2' 5' tetrahydroxy-4'-methoxyflavone (isoetin 4'-methyl ether)

Compound 13 and 14 obtained as yellow powder respectively. UV spectra in MeOH showed λ_{max} of 203.27, 255.27, 355.27 nm for compound 14, and 207.27, 257, 354.27 nm for compound 13 respectively. The ESI–MS peaks of compound 13 and compound 14 in negative mode, were observed at m/z 463 [M-H] - and m/z 479 [M-H] - respectively. The molecular ion of compound13 was 16 mass units smaller than that of compound14, which corresponds to the difference in the number of hydroxyl groups on the B-ring of the flavonol aglycone. The ^{1}H NMR spectrum in deuterated methanol of 13 showed signals for quercetin: δ_H 6.19 (d, J=2.0 Hz, H-6, δ_C 99.17), 6.38 (d, J=2.0 Hz, H-8, δ_C 94.09), 7.57 (d, J=2.1 Hz, H-2', δ_C), 6.86 (d, J=8.9 Hz, H-5', δ_C), and 7.56 (d, J=2.1 Hz, 8.9, H-6', δ_C) and anomeric protons at δ_H 5.192 (s, δ_C 104.14) and 5.22 (s, δ_C 104.14) characteristic of galactopyranose. The ^{1}H NMR of 14 in deuterated methanol at 599.74 MHz revealed the presence of myricetin aglycone with five aromatic proton: δ_H 6.19 (s, H-6, δ_C 98.48), 6.38 (s, H-8, δ_C 93.25), 7.37 (s, H-2', H-6', δ_C 108.52) and similar anomeric protons to those in 13 (appendix 9.9). The ^{13}C NMR and HMBC, HSQC spectra in deuterated methanol at 150 MHz of 13 and 14 were very similar, except for the signals corresponding to the flavonol aglycone (appendix 9.8). The spectral data and correlation with literature information revealed the two compounds to be quercetin-3-O-β-galactopyranoside (Rayyan *et al.*, 2005; Yan *et al.*, 2002) and myricetin-3-O-β-

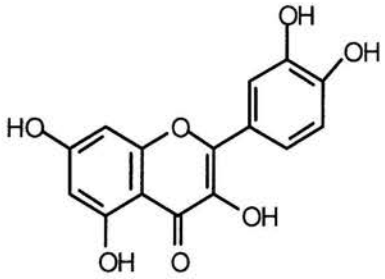
galactopyranoside (Yan *et al.*, 2002) respectively. The chemical structures of the isolated compounds from *Bauhinia galpinii* are presented in Fig. 9.4.



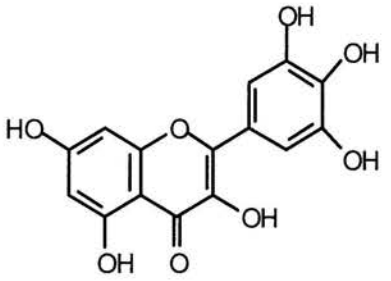
3-beta ethyl sitosterol (9)



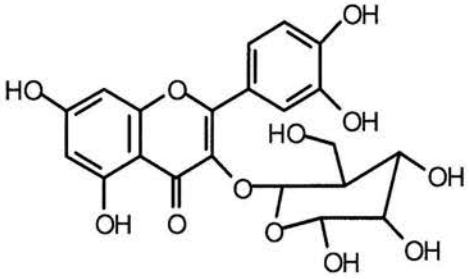
5, 7, 4', 5' tetrahydroxy-2-methoxyflavone
(Isoetin 2'-methyl ether) (10)



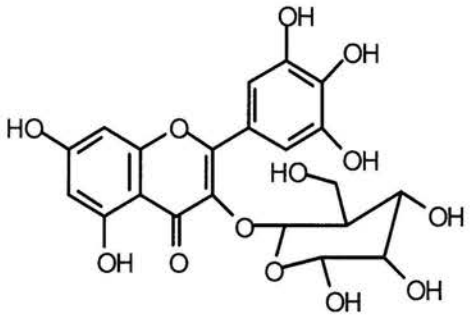
3, 5, 7, 3' 4' Pentahydroxyflavone
(Quercetin) (11)



3, 5, 7, 3' 4', 5' Hexahydroxyflavone
(Myricetin) (12)



Quercetin-3-O-beta galactopyranose (13)



Myricetin-3-O-beta galactopyranose (14)

Fig. 9.4. Chemical structure of bioactive compound isolated from the leaf extract of *Bauhinia galpinii*

9.5.4. Antimicrobial assay of isolated compounds from *B. galpinii*

Antimicrobial potential of the isolated compounds against some diarrhoeal pathogens and organisms of other important infectious diseases are presented in Table 9.2 as minimum inhibitory concentration (MIC) (µg/ml).

Table 9.3. Minimum inhibitory concentration (µg/ml) of the isolated compounds from the leaf extract of *Bauhinia galpinii* and positive control

Microorganisms	9	10	11	13	14	Gentamicin	Amphoteric B
E. coli	125	31	31	31	62	15.5	-
E. faecalis	31	31	7.8	62	62	7.75	-
S. aureus	62	62	15	31	31	3.87	-
P. aeruginosa	125	62	15	15	15	3.87	-
C. albicans (M0825)	15	3.9	3.9	31	31	-	3.9
C. albicans (M0824)	31	31	31	31	31	-	3.9
C. albicans (1051604)	3.9	3.9	3.9	3.9	3.9	-	0.8
C. albicans (1051608)	62	3.9	3.9	15	15	-	1.93
C. albicans (ATCC 10231)	62	3.9	3.9	31	31	-	1.93
C. neoformans	125	31	31	125	125	-	7.75
A. fumigatus	125	31	31	125	125	-	7.75

1: 3β-ethyl sitosterol; 2 Quercetin; 3 Myricetin, 4 Quercetin-3-O-β-galactopyranose; 5 Myricetin- 3-O-β-galactopyranose. *C. albicans* (M0824), *C. albicans* (M0825), *C. albicans* (1051604), *C. albicans* (1051608) are clinical isolate obtained from National Health Laboratory Service, Pretotia, South Africa.

9.6. Discussion

9.6.1. Bioactive compounds from *Combretum vendae*

Bioactivity guided investigation of *Combretum vendae* afforded ursolic acid ((3β)-3-hydroxyurs-12-en-28-oic acid), mixture of asiatic and arjunolic acid, mixture of maslinic and corosolic acid, apigenin, Combretastatin B5-2'-O-β-glucopyranoside and Combretastatin B1-2'-O-β-glucopyranoside all exhibiting broad based microbial growth inhibitory potentials.

Ursolic acid and its derivatives biosynthetically derived from the cyclization of squalene (and other triterpene acids) have been extensively studied as pharmacological active molecules in many *in vitro* and *in vivo* studies. Some of the biological activities include antioxidant, hepatoprotective, anti-inflammatory, anticancer, anti-HIV, vasorelaxant (Aguirre-Crespo *et al.*, 2006) and antidiabetic activities. Several mechanisms have been proposed to explain its anti-inflammatory activity, including inhibition of secretory PLA_2 enzymes, IL-1β secretion, iNOS and COX-2. Ursolic acid have no antispasmolytic effect (Estrada-Soto *et al.*, 2007), however the broad base antimicrobial activities (3.9-125 µg/ml) obtained in this work and other reports of antioxidant, anti-inflammatory and antidiabetic provide pharmacological bases for further investigation of the compound as antidiarrhoeal agent.

Biological activities such as α-glucosidase inhibition of arjunolic acid (18.63±0.32 µg/ml), asiatic acid (30.03±0.41 µg/ml), maslinic acid (5.52±0.19 µg/ml) and corosolic acid (3.53±0.27 µg/ml) isolated from *Lagerstroemia speciosa* were reported. The α-glucosidase inhibition of these compounds shows their antidiabetic and antiadhesion potential against microbial pathogens both of which are also important in antidiarrhoea therapy. However the compounds have no α-amylase inhibitory activities (Hou *et al*., 2009). Mixtures of arjunolic acid and asiatic acid isolated from Combretum nicholsonii have antifungal activities with MIC of 0.2-1.5 µg/ml. However, antimicrobial activities obtained in the work is slightly higher for the mixture (3.9-250 µg/ml), the difference in result may be due to experimental variable such as concentrations of the culture media, composition of the mixture, incubation time and strains. These results indicate that individual compound or the mixtures have pharmacological potentiality against infectious pathogens.

Apigenin have been isolated previously from the acetone leaf extract of *C. vendae*. The antibacterial activity of the compound was evaluated (Eloff *et al*., 2008).

Combretastatin B5-2'-O-β-glucopyranoside have been isolated previously from the seed of *Combretum kraussii*. The aglycone moiety of this compound was isolated from the acetone leaf extract of *Combretum woodii* reported to have antibacterial activity. This is the first report on the isolation of the compound from the leaf extract and its antimicrobial activities.

Combretastatin B1-2'-O-β-glucopyranoside have been previously isolated from the seeds of *Combretum kraussii* (Pettit *et al*, 1987) and wood bark of *Combretum erythrophylum* (Schwikkard *et al*., 2000). The stilbenes have been reported to have cytotoxic activity with effect on tubulin polymerization, the primary protein component of microtubules in cancer hence the potential of the compound as anticancer drug is being explored. The compound has been evaluated for selective inhibitory activity against the DNA-damaging repair-deficient strain of *Saccharomyces cerevisiae* deficient in the RAD52 recombination repair gene and exhibited no activity while the derivative with unsaturated bond at 1aC- 1a'C (combretastatin A1-2'-O-β-glucopyranoside) was active (Schwikkard *et al*., 2000). This indicated the importance of the unsaturated bond in the structure-activity relationship for cytotoxicity effects. However, there is no literature report on other biological activity potential such as antimicrobial, antioxidant, anti-inflammatory and antidiarrhoea. This is the first report on the isolation of the compound from the leaf extract and its antimicrobial activities. The non-cytotoxic effect of the compound against the cancer cells lines ((Schwikkard *et al*., 2000) indicates that the compound can be exploited for other biological activities.

9.6.2. Bioactive compounds from *Bauhinia galpinii*

Isoetin (5, 7, 2' 4' 5'-pentahydroxyl flavone) and its various derivatives are rare compounds formed by insertion of a 2' hydroxyl group into luteolin to give characteristic yellow pigments in some plant part Lattanzio *et al.*, 2006). 5, 7, 4' 5' tetrahydroxy-2'-methoxyflavone (isoetin 2'-methyl ether) is a new compound (unfortunately the quantity obtained was not enough for bioassays). A related compound 5, 7, 2' 4' tetrahydroxy-5'-methoxyflavone (isoetin 5'-methyl ether) isolate from *Trihosanthes kirilowii* (Cucurbitaceae) was reported to be cytotoxic against human lung cell line A549 (IC_{50} 0.92 µg/ml), human melanoma Sk-Mel-2 (IC_{50} 8.0 µg/ml), and mouse melanoma B16F1 cell lines (IC_{50} 7.23 µg/ml). High cytotoxicity (IC_{50} 2.5 µg/ml) of the acetone root extract of *B. galpinii* against Vero cell lines has been reported (Samie *et al.*, 2009). Isolation of more 5, 7, 4' 5' tetrahydroxy-2'-methoxyflavone (isoetin 2'-methyl ether) from *B. galpinii* for further studies on cytotoxicity effect important.

Quercetin and myricetin are the common flavonol present in dietary and constitute the active component of medicinal plant with characteristic hydroxyl substitutions at the 3, 5, 7, 3', 4' (Quercetin), and 3, 5, 7, 3', 4' and 5' (myricetin) positions of flavone ring. The compounds occur in nature mostly as glycoside with D-glucose, L-rhamnose, D-galactose or arabinose rather than free aglycone. Biological effects of phenolic compounds depend on their bioavailability which is determined by the lipophilicity of each molecule. The mechanisms involved in digestion and absorption of phenolic compound is complex but passive transports through the membrane have been proposed. The glycosides and methylated phenolics are not readily absorbed in native form but need to be hydrolysed by intestinal enzymes or colonic microflora to aglycone before absorption.

Quercetin and myricetin alongside their galactopyranoside derivatives were isolated from the acidified 70% acetone leaf extract of *Bauhinia galpinii.* Quercetin galactopyranose and myricetin galactopyranose were previously isolated from this plant and their antioxidant activity and cytotoxicity was evaluated (Aderogba *et al.*, 2007).

A wide range of biological activities related to diarrhoeal pathogenesis including antimicrobial (Naz *et al.*, 2007), anti-inflammatory and spasmolytic due to their antioxidant and/or free radical scavenging (Aderogba *et al.*, 2007) as well as ability to interfere with several enzymatic pathways have been reported for the compounds. Quercetin and myricetin are active against microorganisms of the genera *Bacillus*, *Corynebacterium*, *Salmonella*, *Shigella*, *Staphylococcus*, *Streptococcus*, and against *Escherichia coli* and *Vibrio cholerae* (Naz *et al.*, 2007).

Both compounds also have protective effects on intestinal TJ barrier function through interaction with intracellular signaling molecules, tyrosine kinases and protein kinase C δ (PKCδ) (Suzuki and Hara, 2010). The intercellular TJs are the major determinant of the intestinal physical barrier regulating the paracellular movement of ions, solute, and water through the intestinal epithelium. Impaired intestinal TJs functions are involved in several

intestinal and metabolic diseases, such as diarrhoea, inflammatory bowel disease and food allergy (Suzuki and Hara, 2010). Myricetin inhibits the generation of MDA a cytotoxic by-product of lipid peroxidation of arachidonic acid liberated from membrane phospholipids (Robak *et al*., 1986).

Myricetin has potential as an antiviral agent by its ability to inhibit the reverse transcriptase from Moloney murine leukaemia virus, Rauscher murine leukaemia virus and human immunodeficiency virus (Ono *et al*., 1990). Myricetin also has antidiabetic activity with ability to stimulate lipogenesis and enhanced glucose uptake into adipocytes. The mechanisms postulated include changes in lipid-protein interaction or increase membrane fluidity. Myricetin inhibits the intraluminal accumulation of fluid and prevent diarrhoea induced by castor oil.

9.7. Conclusion

All the isolated compounds from the two plant species have biological activities with relevance against one or more diarrhoeal pathophysiology. The antimicrobial of some of the compounds are worthy of pharmacological consideration. In order to exploit the full potentials of these compounds some *in vitro* and *in vivo* studies are required to determine the mechanism of action.

CHAPTER TEN

General conclusion and future prospects

10. Introduction

Diarrhoea is one of the major health challenges facing the world and especially developing countries. The problem is aggravated due to the increasing number of immunocompromised people infected by HIV, with associated opportunistic infections and other health complications manifesting as diarrhoeal symptoms. The emergence of more virulent strains resulting from drug resistant pathogens and the apparent side effects of some conventional drugs currently in use is also serious concerns in diarrhoeal control and management. In animal production, diarrhoeal outbreaks usually cause serious economic losses due to reduced productivity, cost of treatment, lower level of reproduction and increased mortality.

However, the success of oral rehydration therapy in reducing mortality and lack of commercial interest in drugs for developing countries has slowed the progress in the development of novel agents for treating diarrhoeal diseases. Therefore, there is an urgent need for new therapeutic drugs or herbal products with lower cost, high efficacy and little or no side effects. Plants and plant preparations have been used ethnopharmacologically in treating diarrhoea successfully, although their efficacies, mechanisms of action and safety have generally not been proven scientifically. Thus, the overall aim of this project was to systematically determine the efficacy, mode of action and safety of some plants used traditionally in South African traditional medicine as diarrhoea therapy. The following objectives were identified to attain this aim:

- To conduct comprehensive literature works on diarrhoeal aetiologies and mechanisms and, medicinal plants use for treating diarrhoea symptoms in Southern Africa.
- To determine the phenolic compositions of the crude extract.
- To evaluate the effects of some selected medicinal plant species against pathogenic microbes known to induce diarrhoea.
- To determine the antioxidative properties of the selected plants using various standard protocols.
- To determine the anti-inflammatory potentials of the selected plants using various standard protocols.
- To evaluate the toxicity risk of the crude extracts.
- To determine the intestinal motility modulatory effects of the most promising extracts on the contractility process of the isolated rat ileum induced by spasmogens and ion channels activators.
- To isolate and characterize the component(s) that exhibit antimicrobial and antioxidant properties from the most promising extracts.

The achieved objectives of the study are outlined as follows:

10.1. Identification of diarrhoeal pathogenesis and medicinal plants used as therapeutic agents

The data generated from the literature work indicate that diarrhoea has a number of pathogenesis such as microbial infection, chronic inflammation, oxidative injury to intestinal mucosal lining, and deranged intestinal motility. The mechanisms involved include one or combination of ionic and water secretion into the lumen and reduced absorption of fluid from the intestine. The compendium of the medicinal plants used as antidiarrhoeal agents (254 species) in Southern Africa also revealed the diarrhoeal challenges and the wide acceptability of medicinal for cure.

10.2. Antimicrobial evaluation of the extracts against infectious pathogens

The results obtained by the antimicrobial screening indicate the presence of many compounds with potent antibacterial and antifungal activity against the standard strains of microbes responsible for infectious diarrhoea and other important infectious diseases in humans and animals. The significant inhibitory activity exhibited by the water fraction of the *C. vendae* against *S. aureus* can be considered as important for the traditional use of this plant, where water is the main extractant available. Generally, the results revealed that the antimicrobial potential of the extracts are potentiated in the hexane and dichloromethane fractions. Future investigation of the potent extracts and fractions against resistant and virulent pathogens might indicate new mechanisms for the growth inhibition of the microorganisms.

10.3. Antioxidant evaluation of the extracts

The crude extracts and the polar fractions of ethyl acetate and butanol had significant radical scavenging activity, through hydrogen and proton donation mechanisms. These activities are ascribed to the presence of large quantity of phenolics. In view of the oxidative stress in the pathogenesis of diarrhoea through tissue injury by ROS/RNS involvement in lipid peroxidation, exacerbation of inflammatory processes and some of the reactive species serving as secretagogues, the strong antioxidant activity could indicate the presence of compounds with potentially important mechanisms of pharmacological relevance in reducing the deleterious effects of the oxidative species in diarrhoea. *In vitro* results however, cannot be literally translated into *in vivo* situation due the problem of bioavailability, absorption and possible metabolic transformations of the bioactive compounds in the intestine. Further research are needed to verify using other models with different mechanisms against substrates which are generated in human or animal cells as well as *in vivo* studies to evaluate their efficacy and safety.

10.4. Anti-inflammatory potential of the extracts

Inflammation is regarded as the hallmark of many diseases aetiology and the significant mediators are eicosanoids (prostaglandins, prostacyclin) from cyclooxygenase (COX) and leukotrienes from lipoxygenase (LOX) pathways. These two enzymes are the target for modulating the inflammatory process. The result obtained indicated that the polar extracts of *Bauhinia*, *Carissa*, and *Syzygium* species used for COX inhibitory assay were active against COX-1 with no activity against COX-2 while the *Combretum* species were inactive against both enzymes. COX-1 selective inhibitors are considered to cause GIT injury while selective COX-2 inhibitors are more beneficial against inflammatory processes, therefore these plant polar extracts should be used with caution because possible intestinal injury. Most of the plant extracts however, had good LOX inhibitory activity. Current research on anti-inflammatory agent focus on dual activity as COX and LOX inhibitors since both pathways uses the same substrate. If one pathway is closed down, more substrate will be available to the other unperturbed pathway, thus increasing its products and consequently promoting some other inflammatory mechanisms. Additional work is required to determine the fraction(s) in which the active component is present and the probable mechanisms of action. Since the polar extract are not active against COX-2, the non-polar extracts or fractions still have to be tested. The plant extracts also have to be tested against other inflammatory biomarkers and mediators including an *in vivo* studies using laboratory animal model.

10.5. Toxicity risk of the extracts

The toxicity risk assessment using MTT assay (Mosmann, 1983) using Vero African green monkey kidney cell lines indicated that the extracts of *Combretum* species except *C. bracteosum* were highly toxic. The other extracts have varying degree of toxicity with *Ozoroa mucronata* being the least toxic. These results are important because they show that there are risks of toxicity with an inappropriate use of some of these extracts as therapeutics for any ailments except perhaps cancer. The toxicity of medicinal plants depends on many factors such as the plant part used, and the solvents used as extractant which are determinant of the compositional characteristics and biological activity of extracts. Most of the highly toxic extracts also contain a high quantity of hydrolysable tannin. Poor handling of raw or processed materials may produce exogenous toxic contaminants not inherent as plant phytochemicals. It should be kept in mind that the results of cytotoxicity testing may vary considerably depending on the cell type used, the initial cell density to which the extracts are exposed, and the duration of exposure. Vero cells were selected as these are readily available and are commonly used in cytotoxicity tests. In this study, a low cell density was used and the cells were exposed for a long time, 5 days (McGaw et al., 2007). Hence, relatively low LC_{50} values were obtained and differences in cytotoxicity between the extracts were maximised. Further work is needed to test the extracts, fractions and subfractions against other cell lines and, also to conduct acute and chronic toxicity assays with a view of determining the toxic constituents present in the plants.

It is a pity that one of the two plant species selected for in depth work *C. vendae* had a high cellular toxicity. In future studies toxicity of extracts should be investigated at an early stage.

10.6 Motility modulatory effects of *Bauhinia galpinii* and *Combretum vendae*

Considering the wide ethnopharmacological use of *B. galpinii* and *C. vendae* against GIT disorders and their excellent activity in some of the preliminary screening, the two plants were chosen for motility modulatory assays despite the toxicity potential of *C. vendae*. This was with the view that the toxic component(s) will be determined and separated from the other active components. The data generated by the study indicate that *B. galpinii* has a dual-mechanism of action (prokinetic and relaxant) on gastro-intestinal motility while *C. vendae* extracts exhibited spasmolytic (relaxant) effects on isolated rat ileum through multiple mechanisms. These results were important as they indicate that *B. galpinii* extract can clinically be relevant as therapeutic agent in diarrhoea and constipation which are both diseases with aetiology based on motility disturbances to a large extent while the presence of multiple acting spasmolytic activities in the *C. vendae* extract might be contributing towards its effectiveness in diarrhoea and abdominal spasm therapy. Further work are needed for the identification of the specific ENS receptors through which these extract acts as well as the phytochemical compounds responsible for their activities.

10.7. Isolation and characterisation of antimicrobial compounds

Bioassay-guided protocols for antibacterial and antioxidant activity were adopted for the identification and isolation of 14 compounds (8 from *C. vendae* and 6 from *B. galpinii*) using open column chromatography with silica gel and Sephadex LH 20 as stationary phases. However, some of the compounds are mixtures of position isomers which are extremely difficult to separate. The compounds were characterised as ursol-12-en-28-oic acid (ursolic acid), a mixture of corosolic acid and maslinic acid, and a mixture of asiatic acid and arjunolic acid, two stilbenoid glycosides (combretastatin B5-O-2'-β-D-glucopyranoside and combretastatin B1-O-2'-β-D-glucopyranoside) and one flavone (apigenin) from the *Combretum vendae*.

One phytosterol (β-3 ethyl sitosterol), one new flavone (5, 7, 4' 5' tetrahydroxy-2'-methoxyflavone (isoetin 2'-methyl ether) or 5, 7, 2' 5' tetrahydroxy-4'-methoxyflavone (isoetin 4'-methyl ether)), two known flavonols (3, 5, 7, 3', 4'-pentahydroxyflavone (Quercetin) and 3, 5, 7, 3', 4', 5'-hexahydroxyflavone (myricetin)) and their galactoside derivatives (quercetin-3-O-β-galactopyranoside and myricetin-3-O-β-galactopyranoside) were isolated from *Bauhinia galpinii*.

The results from this study indicate that medicinal plants used in ethnopharmacology are reservoirs of bioactive compounds. Some of the medicinal plants may serve as potential sources of novel active compounds or lead molecule for synthesis of more potent drugs. There is also a distinct possibility of developing plants extracts that

could be used by poor rural people or sophisticated herbal medicines from some of the species investigated in this study. The information gained from this work provides a baseline study for other scientist to explore other medicinal plant species in depth with possible commercial application.