CHAPTER THREE

Plant selection, collection, extraction and analysis of selected species

3.1. Introduction

Renewed interest in the therapeutic potential of medicinal plants means that researchers are concerned not only with validating ethnopharmacological usage of plant, but also with identifying, isolating and characterizing the active components (Fennell *et al.*, 2004). However, the presence of numerous inactive components makes the screening and isolation of the target component(s) extremely cumbersome (Sticher, 2008). In choosing medicinal plants for scientific evaluation of their biological activities and validation of ethnopharmacological usage, some criteria such as

- Evidence of ethnopharmacological usage by the native population.
- The ailment(s) which the plant(s) is used to cure.
- The availability of the plant in its natural habitat.
- The sustainable use of the part(s) of the plant (root, leaves, stem, bark or whole plant) (Baker *et al.*, 1995; Van der Watt and Pretorious, 2001).
- Mode of preparation and administration by traditional healers must also be considered.

Plant quality and pre-treatment are also important determinant of the phytochemical constituents and invariably the biological activities of an extract. These factors depend on plant parts used, genetic variation, geographical location, climatic conditions, collection period, drying methods, and storage conditions. Due to these possible variations, plant material from recognized botanical gardens or herbaria is usually recommended because they are protected, correctly identified and serve as reliable sources for subsequent collections. Preparation of voucher specimens is also an important aspect of medicinal plant research. Standard procedures for pre-treatment of plant materials have been developed (Eloff *et al.*, 2008). The basic steps include pre-washing if necessary, air drying under shade at room temperature, grinding into powder and storage in an air tight container at appropriate temperature (room or refrigerated).

In view of limited resources and the large number of potential medicinal plants to be studied, efficient systems of evaluation need to be developed for rapid phytochemical and biological screening. The first step is the use of appropriate extraction process to remove the phytochemical from the plant cellular matrix (Sticher, 2008). Extraction processes need to be exhaustive, efficient, simple, rapid and inexpensive in extracting targeted compound(s). A number of extraction methods such as soxhlet, percolation, maceration, digestion, reflux, and steam distillation have been developed over the years (Sticher, 2008). However, solid-liquid extraction with a suitable range of solvents remains the most viable, convenient and effective procedure widely in use. The plant

extracts are usually qualitatively analyzed for chemical composition (phytochemical fingerprint) and biological activities (for example bioautography for antimicrobial assay, antioxidative profiling with DPPH radical solution, acetyl cholinesterase inhibition) on thin layer chromatography (TLC). These plant pre-treatment methods, extraction and analyses were employed in this chapter to determine qualitatively the phytochemical constituents and biological activities of selected plant extracts. The plants studied were selected based on literature documentation of their use in South African traditional medicine (SATM) as antidiarrhoeal agents and results from preliminary antimicrobial studies on some of the species in the phytomedicine tree project.

3.2. Solid-liquid extraction

Extraction is first pre-purification step in the isolation and characterization of active compound(s) of a medicinal plant (Sticher, 2008). Selective removal of interfering components from solid plant material involves a five-unit operation:

- Mixing of plant material and extractant.
- Solubilisation of the solute with the aid of a shaker or sonicator.
- Filtration of the mixture to remove solutes and extractant from the plant residue.
- Drying of sample using technique such as freeze drying, evaporation under vacuum (rota-evaporation) or air drying.
- Recovery of the solute extract.

The type of extractant may range from non-polar to polar solvent depending on the targeted class of bioactive component(s). Though the method is relatively simple, some of the drawbacks include: long extraction time, labour intensive, high solvent consumption and inadequate reproducibility. In traditional medicine practice, ethanol and water are the most widely used extractants. The bioactive components of medicinal plants are usually unknown, and the nature of the extractant used affects the composition of the crude extract. Therefore, solvents such as hexane, dichloromethane, ethyl acetate, acetone, methanol, propanol, water or a combination of solvents are used in laboratory settings. Acetone has been adjudged to be the best extractant of plant extract for bioassay because it extracts a broad spectrum of components (polar and non-polar), is miscible with all other solvents, is highly volatile, and exhibit low toxicity to biological organisms in various assays (Eloff, 1998).

Temperature is also an important factor in extraction, drying and storage of plant extracts because of varying compound stability due to chemical degradation, losses by volatilization and oxidation. Milder extracting and drying temperatures are required to avoid loss of activity by plant extracts possibly due to thermal decomposition. Storage of plant extracts, fractions or isolated pure compounds should be done at 4^{o}C in the dark to avoid any negative influence of temperature and light.

3.3. Liquid-liquid fractionation

Solvent partitioning of extracts allows a finer separation of the plant constituents into fractions of different polarity. Bioactivity-guided fractionation, where the fractions are tested following separation to quickly identify and isolate the agents responsible for bioactivity is a desirable step in medicinal plant research. The solvent partition process involves the use of two immiscible solvents of different polarities. Various solvents are used starting with non-polar (hexane, dichloromethane, diethyl ether) to medium polar (chloroform, ethyl acetate), and finally more polar solvent (acetone, methanol, butanol and water).

3.4. Thin layer chromatography (TLC)

3.4.1. Phytochemical fingerprints

TLC is widely used in natural product extract analysis, stability tests of extracts and finished products, and in sample quality control (Cimpoiu, 2006). TLC fingerprints of medicinal plants and extracts can be used for identification and quality control of medicinal preparations. The identification of separated components can be achieved on the basis of retention factor (R_f) values and colour spots. In relation to other chromatographic methods, TLC offers the simplest and cheapest means of detecting natural product constituents, requiring little sample clean-up and equipment (Nyiredy and Glowniak, 2001). Characteristic features of TLC include: analysis of many samples and comparison of their phytochemical profiles on the same plate; results can be stored and communicated as images (picture, video or scanned) and flexibility in the choice of mobile and stationary phase (Cimpoiu, 2006). Identification of compounds can be done using three different mobile phases on the same stationary phase or three different stationary phases with one mobile phase to develop the fingerprint of the extracts and standards. If the difference in R_f values is less than 0.03, then the compounds is identified without further isolation (Nyiredy and Glowniak, 2001). However, position isomeric compounds such as ursane and oleanane derivatives can have superimpose or close R_f values, making them inseparable.

Visualization of separated compounds is achieved by natural colour in daylight or by fluorescent quenching on 254 nm (for conjugated double bonds or extended π electron systems) or 366 nm UV light. Some commercial plate absorbents contain fluorescent dye that lights when placed under UV light and compounds are indicated with blue, green, brown, red or purple areas against a fluorescent background. Visualization of chromatogram under UV light at 366 nm shows orange-yellow bands for flavonoids and blue fluorescent bands for phenolic acids (Males and Medic-Saric, 2001). Many chromogenic spray reagents are also available for specific classes of compounds or serve as indicators for broad classes of compounds. Examples are vanillin/sulphuric acid solution, anisaldehyde and ferric chloride-potassium ferricyanide given intense blue bands for phenolic compounds (Wettasinghe *et al.*, 2001).

3.5. Materials and Methods

3.5.1. Selection of South Africa medicinal plants for antidiarrhoeal screening

For this project, 27 plant species from nine families (Table 3.1) were selected for preliminary screening based on the following criteria:

1. Ethnopharmacological use of the plant in the management of diarrhoea locally,
2. Phylogenetic relationship to other plants used in treatment diarrhoea due to the possibility of their producing related chemical compounds (chemotaxonomy),
3. Medicinal plants reportedly used in countries other than South Africa but naturalized or endogenous in South African flora,
4. Preliminary pharmacological evaluation of the medicinal plant from the phytomedical laboratory of the Department of Paraclinical Sciences (University of Pretoria),
5. Absence of published literature describing antidiarrhoea and biological studies, and
6. Their availability for evaluation.

A literature review on the selected plants for antidiarrhoea and other biological studies yielded little or no previous research work.

3.5.2. Collection of plant materials

The leaves of the 27 plants were collected from the Marie van der Schijff Botanical Garden University of Pretoria Main Campus at Hatfield, Pretoria or from Phytomedicine Programme tree project stored samples. The plants were identified and authenticated by Ms. Lorraine Middleton and Magda Nel at the University of Pretoria Botanical Garden. Voucher specimens were maintained at the HGWJ Schweikert Herbarium of the Department of Plant Science, University of Pretoria, Hatfield Campus, Pretoria, South Africa.

3.5.3. Preparation of plant material and optimization of phenolic-enriched extraction process

Plant leaves collected were pre-treated according to Phytomedicine programme (University of Pretoria) standard protocol. In brief, the leaves were sorted from the stem, packed in a well perforated bag and air dried under shade at room temperature for 2 week. The dried leaves were ground, powdered and kept in an air tight polyethylene bag until needed for the extraction process. Simultaneous extraction and fractionation of the leaves using a mixture of 70% acetone acidified with 0.1% HCl and hexane. The chlorophyll, fat and wax-enriched hexane fraction was decanted from the phenolic-enriched 70% acetone fraction.

Table 3.1: Medicinal plants selected for antidiarrhoeal investigation in this study

Genera	Family/Species	syn	Voucher specimen information	Reasons for selection
Anacardiaceae	*Ozoroa mucronata* (Bernh.ex C.Krauss) R.fern & A. Fern	Ozm	PRU 068928	2, 4, 5, 6
	Ozoroa paniculosa (Sond.) R.fern & A. Fern	Ozp	PRU 66851	1, 2, 4, 5, 6
	Searsia leptodictya Diels	Sle	PRU 70151	2, 4, 5, 6
	Searsia pendulina Jacq.	Spd	PRU 84141	2, 4, 5, 6
	Searsia pentheri Zahlbr.	Spt	PRU 709769	2, 4, 5, 6
Apocynaceae	*Carissa macrocarpa* (Eckl.) A.DC	Cam	PRU 37819	2, 4, 5, 6
Burseraceae	*Commiphora harveyi* (Engl.) Engl.	Com	PRU 49952	2, 4, 5, 6
Celastraceae	*Maytenus peduncularis* (Sond.) Loes.	Mpd	PRU 76382	2, 4, 5, 6
	Maytenus probumbens (L.f.) Loes.	Mpr	PRU 77119	2, 4, 5, 6
	Maytenus senegalensis (Lam.) Exell	Mse		1, 2, 3, 4, 5
	Maytenus undata (Thunb.) Blakelock	Mun	PRU 18576	1, 2, 3, 4, 5, 6
Combretaceae	*Combretum bracteosum* (Hochst.) Brandis ex Engl.	Cob	PRU 117443	1, 2, 4, 5, 6
	Combretum padoides Engl. & Diels	Cop	PRU 115416	1, 2, 4, 5, 6
	Combretum vendae A.E. van Wyk	Cov	PRU 50800	1, 2, 4, 5, 6
	Combretum woodii Dummer	Cow	PRU 20544	1, 2, 4, 5, 6
Ebenaceae	*Euclea crispa* (Thunb.) Gurke	Euc	PRU 76444	2, 4, 5, 6
	Euclea natalensis A.DC.	Eun	PRU 66327	1, 2, 4, 5, 6
Fabaceae	*Bauhinia bowkeri* Harv	Bab	PRU 44967	2, 4, 5, 6
	Bauhinia galpinii N. E. Br	Bag	PRU 28944	1, 2, 4, 5, 6
	Bauhinia petersiana Bolle	Bap	PRU 66874	2, 4,5
	Bauhinia variegata L.	Bav	PRU 38533	1, 2, 3, 4, 5, 6
	Erythrina latissima E. Mey	Erl	PRU 16349	2 , 4, 5, 6
	Indigofera cylindrical sensu E. Mey	Inj		2, 3, 4, 5, 6
	Schotia brachypetala Sond.	Scb	PRU 55333	1, 2, 4, 5, 6
Moraceae	*Ficus craterostoma* Warb.ex Mildbr. & Burret	Fic	PRU 38554	2, 4, 5, 6
	Ficus glumosa Delile	Fig	PRU 48293	1,2, 4, 5
Myrtaceae	*Syzygium paniculatum* Gaertner	Syp	PRU 115417	2, 3, 5, 6

(1) Ethnopharmacological use of the plant in the management of diarrhoea locally, (2) phylogenetic relationship to other plants used in treatment diarrhoea due to the possibility of their producing related chemical compounds (chemotaxonomy), (3) medicinal plants reportedly used in countries other than South Africa but naturalized or endogenous in South African flora, (4) preliminary pharmacological evaluation of the medicinal plant from the Phytomedicine Programme of the Department of Paraclinical Sciences (University of Pretoria), (5) absence of published literature describing antidiarrhoea and biological studies, and (6) their availability for evaluation.

The acetone residue was removed by evaporation under vacuum using a rotary evaporator at 40°C. The residual water fractions were divided into two portions (A and B). Portion A was freeze dried and served as the crude extract while portion B was fractionated using solvents of increasing polarities as presented in Figure 3.1. The crude extracts and fractions were reconstituted in various suitable solvents for the biological assays.

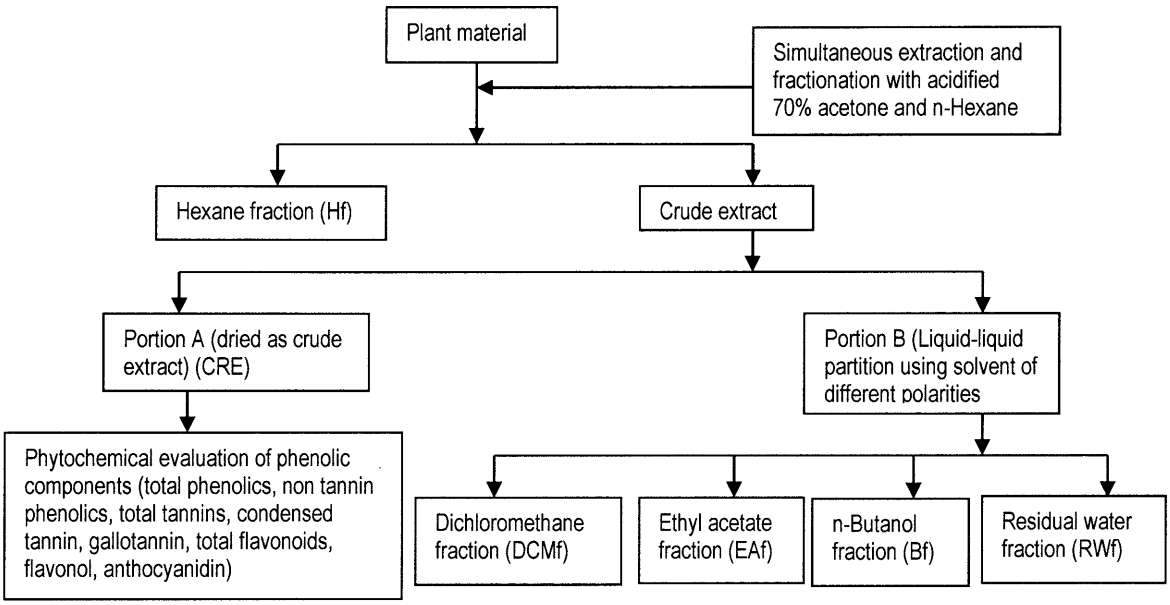

Fig. 3.1. Flow chart for the extraction, phytochemical analysis and fractionation of plant material

3.5.4. Phytochemical profiling

The phytochemical profiles of the crude extracts and fractions were determined using thin layer chromatography (TLC) by spotting 10 µl of solution at a concentration of 10 mg/ml. The plates were developed with various combinations of hexane (H), ethyl acetate (E), formic acid (F), acetic acid (A), chloroform (C), methanol (M), water (W), benzene (B) and ammonia (Am) at different ratio to create eluting solvent of varied polarities. The combination with ratios in parenthesis that were used:

(1) E: F: A: W (70:5:5:10)
(2) E: F: A: W (70:5:15:10)
(3) E: M: Am (90:20:15)
(4) H: E: F (90:10:2),
(5) H: E: F (70:30:2)
(6) H: E: F (50:50:2)
(7) (B: E: Am (90:10:1)
(8) C: E: F (50:40:10)

(9) E: M: W: F (50:6.5:5:2)

(10) H: E: F (20:80:2)

The developed TLC plates were sprayed with vanillin/H_2SO_4 solution and heated at 100°C to allow colour development (FAO/IAEA, 2000). Other reagents such as ferric chloride-potassium ferricyanide and p-anisaldehyde/H_2SO_4 (acetic acid, 5 ml; conc. H_2SO_4, 25 ml; ethanol, 425 ml; water, 25 ml) (Kubata *et al.*, 2005) were also used.

3.6. Quantification of the phenolic constituents of the extracts

3.6.1. Determination of total phenolic constituents

The total phenolic composition of the extracts was determined using the Folin-Ciocalteau method as described by Makkar (2003), with some modifications. Crude extracts (50 µl) at a concentration of 1.0 mg/ml were dispensed into a test tube and made up to 500 µl with distilled water. Subsequently, 250 µl of commercial Folin-Ciocalteau reagent diluted with distilled water (1:1) and 1250 µl of 20% sodium carbonate solution were added to the extract. The mixture was vortexed and the absorbance recorded at 725 nm (using a Versamax microplate reader) after 40 min at room temperature. Total phenolic content (expressed as mg gallic acid/g dry weight) was calculated from the standard curve (0.0019 – 0.25 mg/ml gallic acid) using the following best fit equation: Ac= 4.9022 × TP (mg GAE), R^2=0.98, where Ac is the absorbance of the extracts in presence of an indicator and TP is the total phenolics.

3.6.2. Determination of total tannin

The total tannin content of the extracts was determined using polyvinylpyrrolidone (PVPP) binding method (Makkar, 2003). The bound mixtures were prepared by mixing 100 mg of PVPP, 1.0 ml of distilled water and 1.0 ml of tannin-containing extracts in a centrifuge tube. The mixtures were mixed thoroughly and kept at 4°C for 15 min and then filtered. The filtrate (100 µl) was transferred into a test tube and the phenolic content was evaluated as described in section 3.6.1 above. Non-tannin phenolic constituents were determined from the standard curve of catechin expressed as catechin equivalent in mg/g dry material. The standard curve equation is y = 4.9022x + c, where y is absorbance, x is mg Gallic acid, c=0, R^2=0.9804). The tannin content was calculated as the difference between the total phenolic and non-phenolic content of the extracts because the tannin was bound and precipitated by PVPP.

3.6.3. Determination of proanthocyanidin

The proanthocyanidin content of the extracts was determined using the butanol-HCl assay as described by Makkar, 2003. The extract (500 µl) was dispensed into a test tube and diluted to 10 ml with 70% acetone. Three ml of the 95/5 butanol/HCl reagent and 100 µl of 2% ferric ammonium sulphate in 2N HCl were added. The test

tubes were loosely covered and heated in a boiling water bath for 50 min. The absorbance was recorded at 550 nm (using a Versamax microplate reader) after the tubes and contents were cooled to room temperature. Absorbance of the unheated mixture was used as blank.

3.6.4. Determination of condensed tannin

The condensed tannin content of the extracts was determined using the vanillin/HCl assay described by Heimler *et al.*, 2006. To 0.5 ml of the extract measured into a test tube, 3 ml of vanillin reagent consisting of 4% concentrated HCl and 0.5% of vanillin in methanol was added. The mixture was allowed to stand for 15 min. The absorbance was recorded at 500 nm (using a Versamax microplate reader) against methanol as the blank. The condensed tannin content of the extracts expressed as mg catechin equivalent per gram (mg CE/g) dry plant material was calculated from the standard curve ranged from 0.0019 to 0.25 mg/ml using the following equation: A = 0.1791 × (CT) mg CE + 0.0504, R^2=0.94, where A is the absorbance of the extracts in presence of an indicator and CT is the condensed tannin content.

3.6.5. Determination of hydrolysable tannin (gallotannin)

The gallotannin content of the extracts was determined with the potassium iodate assay (Vermerris and Nicholson, 2006). To 3 ml of the extract, 1 ml of saturated solution of potassium iodate was added and allowed to stand at room temperature for 40 min. The absorbance was read at 550 nm (using a Versamax microplate reader).Triplicate analyses were conducted and the mean values and standard deviations were calculated. A standard curve was prepared using gallic acid under the same conditions as the extracts and results expressed as gallic acid equivalent (GAE)/g dry plant material using the following best fit equation: A = 0.8264 × GT (mg GAE) + 0.0392, R^2=0.92, where A is the absorbance of the extracts in presence of an indicator and GT is the gallotannin content of the extract.

3.6.6. Determination of total flavonoids and flavonol

The total flavonoid content of the extracts was determined by the aluminium chloride method described by Abdel-Hameed *et al* (2009) with some modification. Briefly 100 µl of the extract was mixed with 100 µl of 20% $AlCl_3$ and two drops of glacial acetic acid. The mixture was diluted with methanol to 3000 µl. Absorbance was read at 415 nm with the Versamax microplate reader after 40 min. Blank samples were prepared with the extract without $AlCl_3$. A standard curve was prepared using quercetin (3.9-500 µg/ml) in methanol under the same conditions. The total flavonoid content of the extracts expressed as mg quercetin equivalent/g of dry plant material was calculated from the best fit curve using the following equation: A = 4.9747 × mg quercetin, R^2=0.9846, where A is the absorbance of the extracts in presence of an indicator and TF is the total flavonoid content.

The flavonol content of the extracts was also determined by the aluminium chloride method described by Abdel-Hameed *et al* (2009) with some modification. The extract (1 ml) was mixed with 1 ml of 20 mg/ml $AlCl_3$ and 3 ml of 50 mg/ml of CH_3COONa. A standard curve was prepared using quercetin (0.0019 – 0.0312 mg/ml) in methanol under the same conditions. Absorbance was read at 440 nm (using a Versamax microplate reader) after 2.5 h. The flavonol content of the extracts expressed as mg quercetin equivalent/g of dry plant material was calculated from the best fit curve using the following equation: A = 34.046 × mg quercetin, R^2=0.9853, where A is the absorbance of the extracts in presence of an indicator and FLL is the flavonol content.

3.6.7. Determination of anthocyannin

The total anthocyanin content of the extracts was determined by a pH differential method with 96-well microplate (Lee *et al*, 2008; Lee *et al*., 2005) using a microplate reader (Versamax). Absorbance was measured at 520 nm and 700 nm in buffers at pH 1.0 and 4.5 using a molar coefficient of 29,600. Results were expressed as mg cyanidin-3-glucoside equivalent/g dry plant material using equations 1 and 2 (Lee *et al*., 2005).

$$A= (A_{520}-A_{700})_{pH\ 1.0} - (A_{520}-A_{700})_{pH\ 4.5} \quad (1)$$

$$\text{Anthocyanin (cyanidin-3-glucose equivalent mg/L)} = A \times MW \times DF \times 10^3/\square \times l \quad (2)$$

Where MW (molecular weight) = 449.2 g/mol for cyanidin-3-glucose (cyn-3-glu); DF = dilution factor; l = path length in cm; □ = 26900 molar extinction coefficient in L × mol^{-1}× cm^{-1} for cyn-3-glu; 10^3 factor for conversion from g to mg.

3.7. Results

3.7.1. Yield of extractions and fractionations processes

The yield of the phenolic-enriched crude extracts and the fractions of various polarities using hexane, dichloromethane, ethyl acetate, butanol and residual water are presented in Table. 3.2. The 70% acetone was an extremely efficient extractant with an average of 34.61±5.84% extracted. The maximum yield was obtained for the crude extracts of *S. leptodicya* (48.50±12.47% g/g dried plant material) followed by *O paniculosa* (43.87±6.60% g/g dried plant material) while *S. pentheri* (21.13±2.67 g/g dried plant material) yielded the least. There was a surprisingly high standard deviation between the three repetitions with a single extraction with new plant material. This may have been caused by a difference in the particle size of samples. The extraction process efficiently removed the chlorophyll from the bulk 70% acetone extractant into hexane fraction. In most cases there was a difference between the percentage extracted and the total percentage of all the fractions. This loss may be ascribed to solubility difficulties encountered with the dried residual water fraction which could not be reconstituted due to the formation of insoluble complexes between the polyphenolics and other high molecular

weight components such as polysaccharides and possibly alkaloids. Unfortunately at that stage a freeze drying was not available. This problem may have been partially resolved if the water fraction was freeze dried. To evaluate the degree to which the different plant species contain compounds of different polarity the percentafe of quantiy present in the crude extract into the different fractions was calculated (Table 3.2).

Table 3.2. The percentage yield of the crude extracts and various fractions (g/g dried plant material)

Plant spp	Crude	Hexane	DCM	ETOAc	Butanol	Residual Water	Insoluble ppt
Bab	33.25±0.83	3.44±0.15	1.39±0.16	2.45±0.34	9.91±1.09	7.47±0.41	8.27±1.87
Bag	38.83±6.18	2.38±0.35	1.31±0.27	2.70±0.21	15.05±1.16	8.93±0.18	10.90±2.94
Bap	32.23±2.84	1.67±0.17	1.71±0.17	3.30±0.78	11.82±0.48	8.71±1.23	7.69±2.53
Bav	31.62±5.46	1.83±0.07	1.90±0.23	2.98±0.17	10.65±1.68	8.07±0.13	
Erl	22.12±0.32	1.35±0.55	0.34±0.05	0.26±0.06	6.63±2.20	10.44±0.96	2.77±1.87
Inc	36.15±1.62	1.58±0.17	0.78±0.08	1.71±0.32	12.10±0.66	9.31±1.51	7.84±2.47
Scb	30.15±3.47	1.53±0.49	1.14±0.40	1.58±0.06	11.54±1.00	7.39±1.93	6.21±1.30
Cob	34.24±3.08	1.27±0.21	3.39±1.13	3.28±0.44	8.15±0.30	8.81±1.40	
Cop	39.96±0.78	2.33±0.51	3.31±0.68	3.56±0.18	17.42±0.79	7.43±1.06	1.84±0.96
Cov	38.77±0.48	1.33±0.47	2.86±0.24	3.13±0.51	14.82±2.53	12.08±0.16	2.74±1.06
Cow	36.88±3.39	3.95±1.75	2.07±0.38	2.67±0.41	12.35±2.99	8.39±0.52	7.41±3.29
Ozm	30.65±2.44	2.0±0.20	0.86±0.11	1.03±0.01	7.28±1.53	14.25±2.08	2.66±1.02
Ozp	43.87±6.60	6.57±0.55	1.55±0.38	4.30±0.82	14.54±0.96	8.81±2.02	8.62±1.77
Sle	48.50±12.47	5.85±0.61	1.49±0.30	4.25±0.52	10.30±1.82	9.29±0.89	13.28±3.34
Spd	33.76±0.28	5.05±0.69	0.98±0.28	3.03±0.35	12.21±0.81	11.40±3.31	1.96±0.53
Spt	21.13±2.67	2.96±0.30	1.50±0.25	1.04±0.28	6.34±0.17	8.80±1.50	1.62±0.89
Mpd	33.12±1.07	3.80±0.04	1.22±0.14	1.32±0.08	8.89±0.92	13.39±1.92	4.24±1.98
Mpr	35.10±4.77	3.05±0.28	1.18±0.31	0.90±0.28	8.50±1.31	12.41±0.47	4.78±1.35
Mse	37.89±3.05	3.75±0.40	1.20±0.14	1.30±0.08	8.77±0.91	13.21±1.89	10.08±2.71
Mun	36.89±4.67	1.42±0.18	1.63±0.48	1.08±0.46	10.88±0.46	12.08±1.77	3.13±1.57
Euc	34.97±1.90	2.76±0.56	1.49±0.32	2.05±0.16	13.44±0.86	10.81±0.30	2.84±0.73
Eun	32.83±3.19	2.05±0.82	1.73±0.21	2.35±0.45	10.89±1.39	12.`6±1.96	2.77±1.87
Fic	25.68±3.22	1.50±0.06	0.63±0.07	0.91±0.07	7.59±1.93	9.39±0.30	2.69±1.36
Fig	35.22±4.04	1.82±0.11	1.13±0.16	1.44±0.17	12.84±1.43	8.35±0.58	10.54±2.55
Cam	40.80±1.57	2.21±0.13	0.90±0.08	2.73±0.42	10.77±2.67	11.04±1.92	9.77±3.54
Com	33.09±1.19	1.01±0.04	1.06±0.28	1.64±0.08	10.39±4.81	7.80±0.59	5.37±1.85
Syp	36.80±8.10	1.44±0.20	0.78±0.06	1.10±0.17	8.34±1.89	10.60±0.40	5.95±2.64

3.7.2. Phytochemical screening (fingerprints)

The TLC phytochemical profiles of the crude extracts and fractions of the 27 plant species investigated are presented in Figs 3.1–3.4. Figures 3.1, 3.2, 3.3, and 3.4 are the TLC fingerprints of the crude, hexane fraction, dichloromethane fraction, ethyl acetate fraction developed with three mobile phases of different polarities for each fraction.

In each chromatogram the order from left to right was **Bab** (*Bauhinia bowkeri*), **Bag** (*Bauhinia galpinii*), **Bap** (*Bauhinia petersiana*), **Bav** (*Bauhinia variegata*), **Erl** (*Erythrina latissima*), **Inc** (*Indigofera cylindrica*), **Scb** (*Schotia brachypetala*), **Cob** (*Combretum bracteosum*), **Cop** (*Combretum padoides*), **Cov** (*Combretum vendae*), **Cow** (*Combretum woodii*), **Ozm** (*Ozoroa mucronata*), **Ozp** (*Ozoroa paniculosa*), **Sle** (*Searsia leptodictya*), **Spd** (*Searsia pendulina*), **Spt** (*Searsia pentheri*), **Mpd** (*Maytenus peduncularis*), **Mpr** (*Maytenus procumbens*), **Mse** (*Maytenus senegalensis*), **Mun** (*Maytenus undata*), **Cam** (*Carissa macrocarpa*), **Com** (*Commiphora harveyi*), **Syp** (*Syzygium paniculatum*).

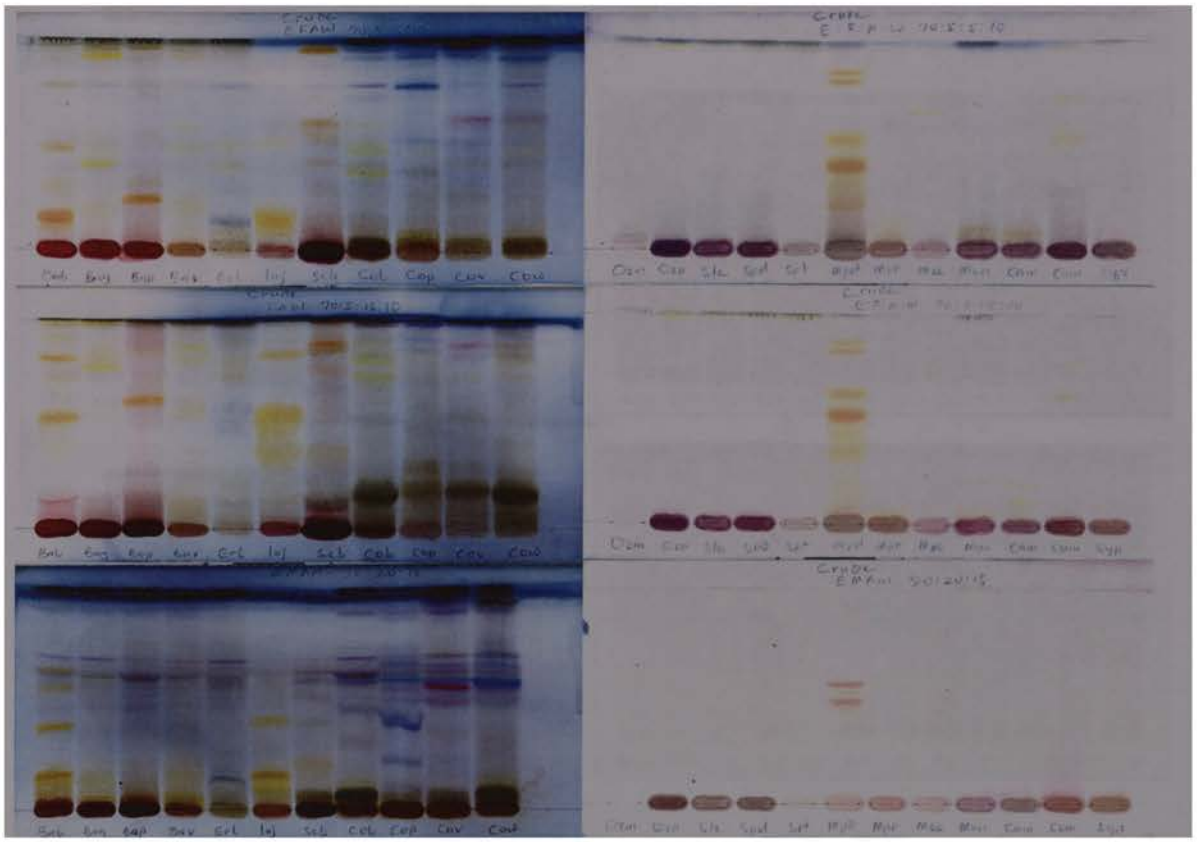

Fig.3.2. Chromatograms of 100 µg of crude extracts of different plant species developed with ethyl acetate: acetic acid: formic acid: water (75:5:5:10) (top), ethyl acetate: acetic acid: formic acid: water (70:5:15:10) (middle) and ethyl acetate: methanol: ammonia (90:20:15) (bottom) and visualized with vanillin sulphuric acid. For identity of plant species see under section 3.7.2 or under abbreviations used.

The chromatograms revealed complex mixture of compounds which exhibited different coloured reactions with the vanillin/H_2SO_4 spray reagent. The classes of compounds in the extracts include terpenoids (purple or bluish purple) (Taganna *et al.*, 2011) and phenolics such as flavonoids (yellow, pinkish or orange), stilbenes (bright red to dark pink colour), and proanthocyanidins (pink colour). The phenolic components were confirmed by blue-black spots with ferric chloride-potassium ferric cyanide reagents (Wettasinghe *et al.*, 2001) while the flavonoids were confirmed by yellow spot (Rijke *et al.*, 2006) with aluminium chloride/acetic acid spray reagent ($AlCl_3$/CH_3COOH).

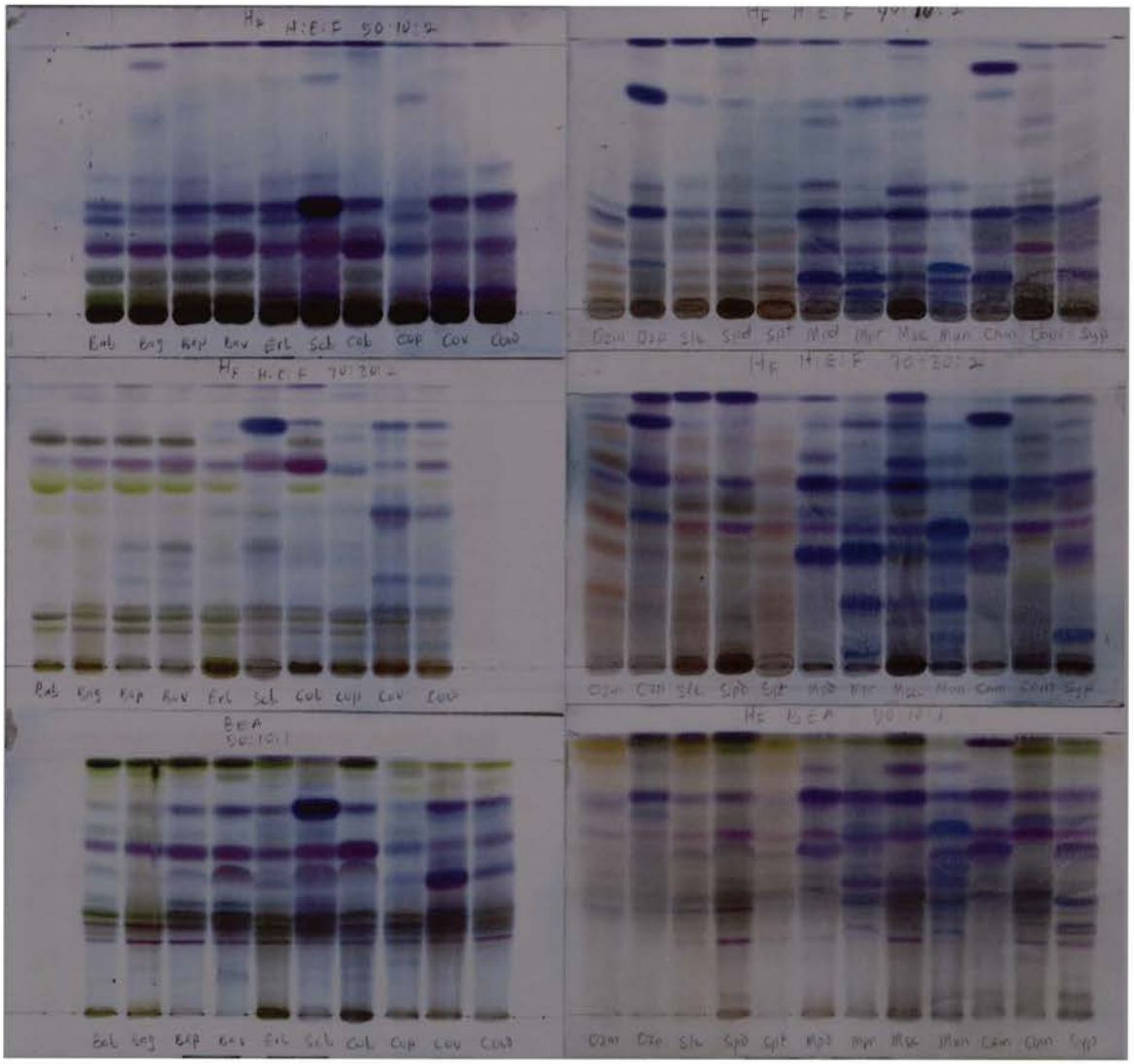

Fig. 3.3: Chromatograms of the hexane fractions of different plant species developed with hexane: ethyl acetate: formic acid (90:10:2) (top), hexane: ethyl acetate: formic acid (70:30:2) (middle), and benzene: ethyl acetate: ammonia (90:10:1) (bottom) and visualized with vanillin sulphuric acid. For identity of plant species see under section 3.7.2 or under abbreviations used.

Characterization of the phytochemical profiles of the extracts indicated that the extraction method and extractants used resulted in splitting the complex mixtures into polar components concentrated in the 70% acetone component (crude extracts) and non-polar compounds concentrated in the hexane component. From the chromatogram, the crude extracts contained phenolics (especially flavonoids and proanthocyanidin) and terpenoids. The hexane and dichloromethane fractions contained prominent spots for terpenoids while the ethyl acetate fractions had prominent spots typical of flavonoid and other phenolic compounds.

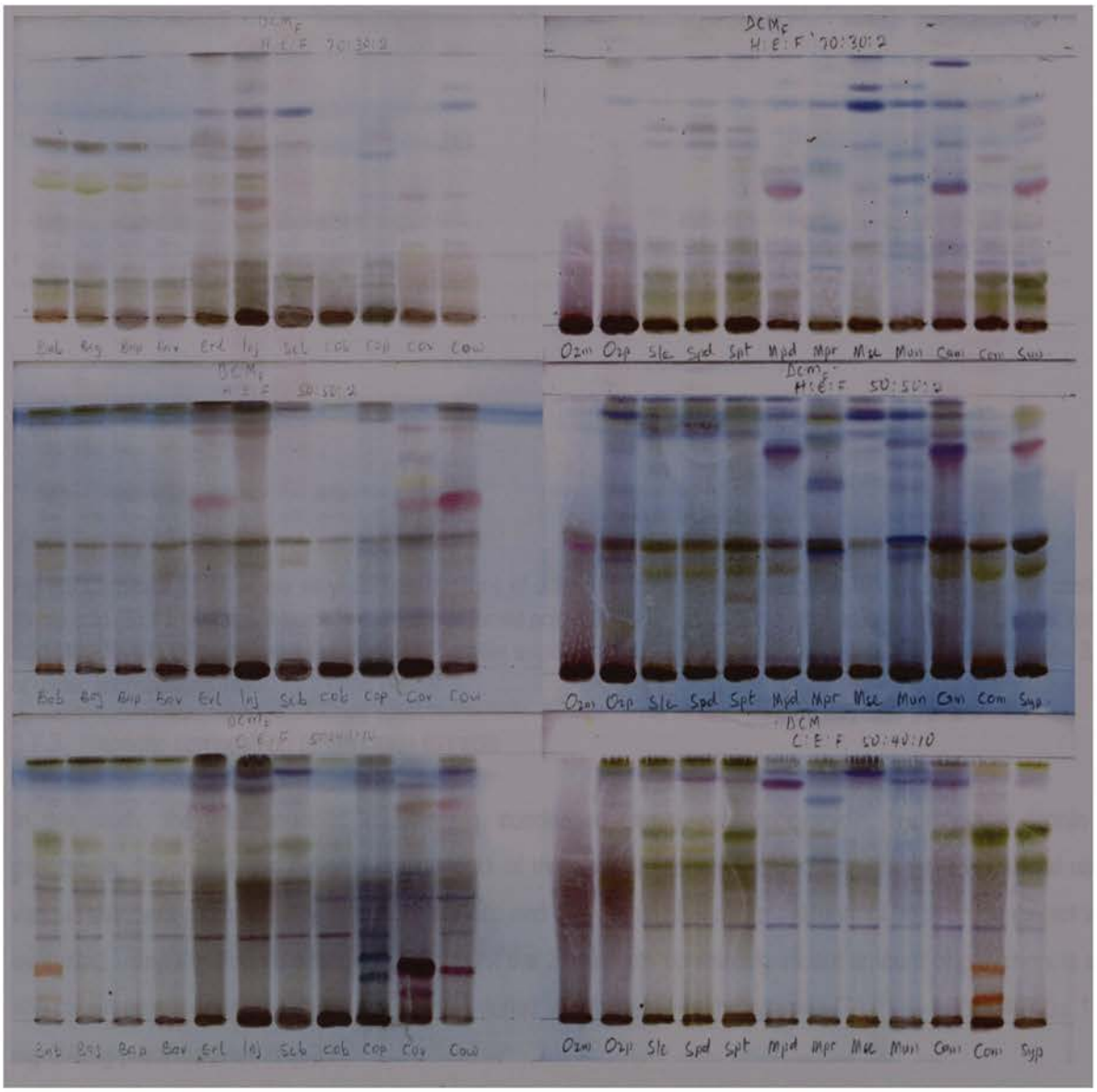

Fig 3.4: Chromatograms of the dichloromethane fractions of different plant species developed with hexane: ethyl acetate: formic acid (70:30:2) top, hexane: ethyl acetate: formic acid (50:50:2) (middle) and chloroform: ethyl acetate: formic acid (50:40:10) bottom and visualized with vanillin sulphuric acid. For identity of plant species see under section 3.7.2 or under abbreviations used.

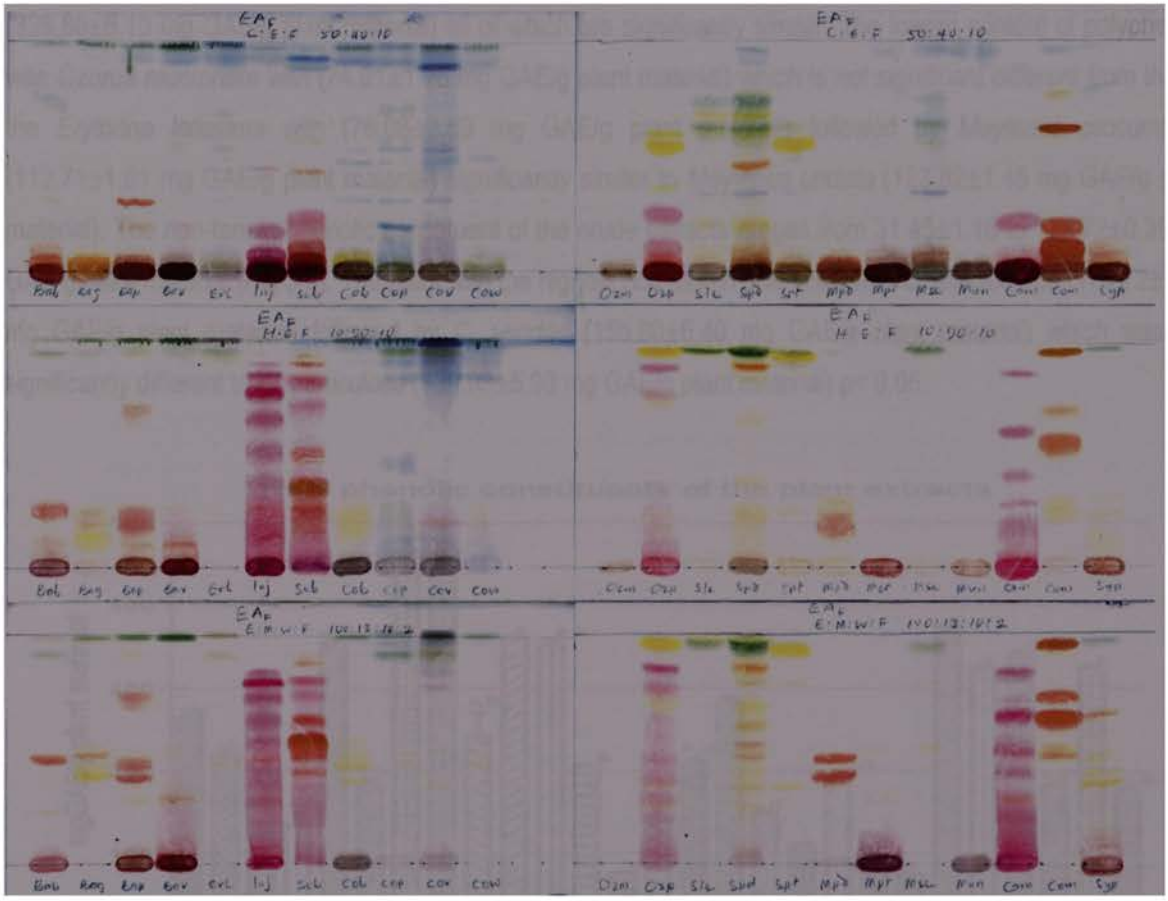

Fig 3.5 Chromatograms of the ethyl acetate fractions of different plant species developed with chloroform: ethyl acetate: formic acid (50:40:10) (top), hexane: ethyl acetate: formic acid (10:90:10) (middle) and ethyl acetate: methanol: water: formic acid (100:13:10:2) (bottom) and visualized with vanillin sulphuric acid. For identity of plant species see under section 3.8.2 or under abbreviations used.

3.7.3. Phenolic composition of the crude extracts

In this study, the total phenolic, total tannin, condensed tannin, proanthocyanidin, hydrolysable tannin as gallotannin, flavonoids and flavonol constituents of the phenolic-enriched crude extracts were evaluated using various standard protocols. The total polyphenolic and non-tannin phenolic constituent of each crude extract was evaluated using the Folin-Ciocalteau reagent. All the 27 extracts contain significant amount of polyphenols and non-tannin compounds; however, the quantity varied widely between the species (74.91 ± 1.26 – 467.0± 15.8 mg GAE/g plant material) (Fig.3.6).

Among the different extracts tested, the highest content of polyphenols was *Combretum padoides* (467.0±15.8 mg GAE/g plant material) which did not differ significantly (P<0.05) to *Combretum vendae* with (444.20±15.4 mg GAE/g plant material). These two plant species were followed by *Carissa macrocarpa* (354.15±3.01 mg GAE/g plant material), *Commiphora harveyi* (362.60±2.10 mg GAE/g plant material), *Euclea natalensis* (204.98±1.89 mg GAE/g plant material), *Ozoroa paniculosa* (370.89±4.80 mg GAE/g plant material) and *Searsia pendulina*

(339.80±5.10 mg GAE/g plant material) all of which are significantly similar. The lowest content of polyphenols was *Ozoroa mucronata* with (74.91±1.26 mg GAE/g plant material) which is not significant different from that of the *Erythrina latissima* with (76.08±2.59 mg GAE/g plant material) followed by *Maytenus procumbens* (112.71±1.51 mg GAE/g plant material) significantly similar to *Maytenus undata* (123.82±1.45 mg GAE/g plant material). The non-tannin phenolic constituent of the crude extracts ranges from 31.45±1.16 to 174.72±0.39 mg GAE/g plant material (Fig 3.6). The plant with the highest non-tannin phenolics was *C. macrocarpa* (174.72±0.39 mg GAE/g plant material) followed by *C. vendae* (155.80±6.40 mg GAE/g plant material) which was not significantly different to *O. paniculosa* (139.93±5.93 mg GAE/g plant material) $p < 0.05$.

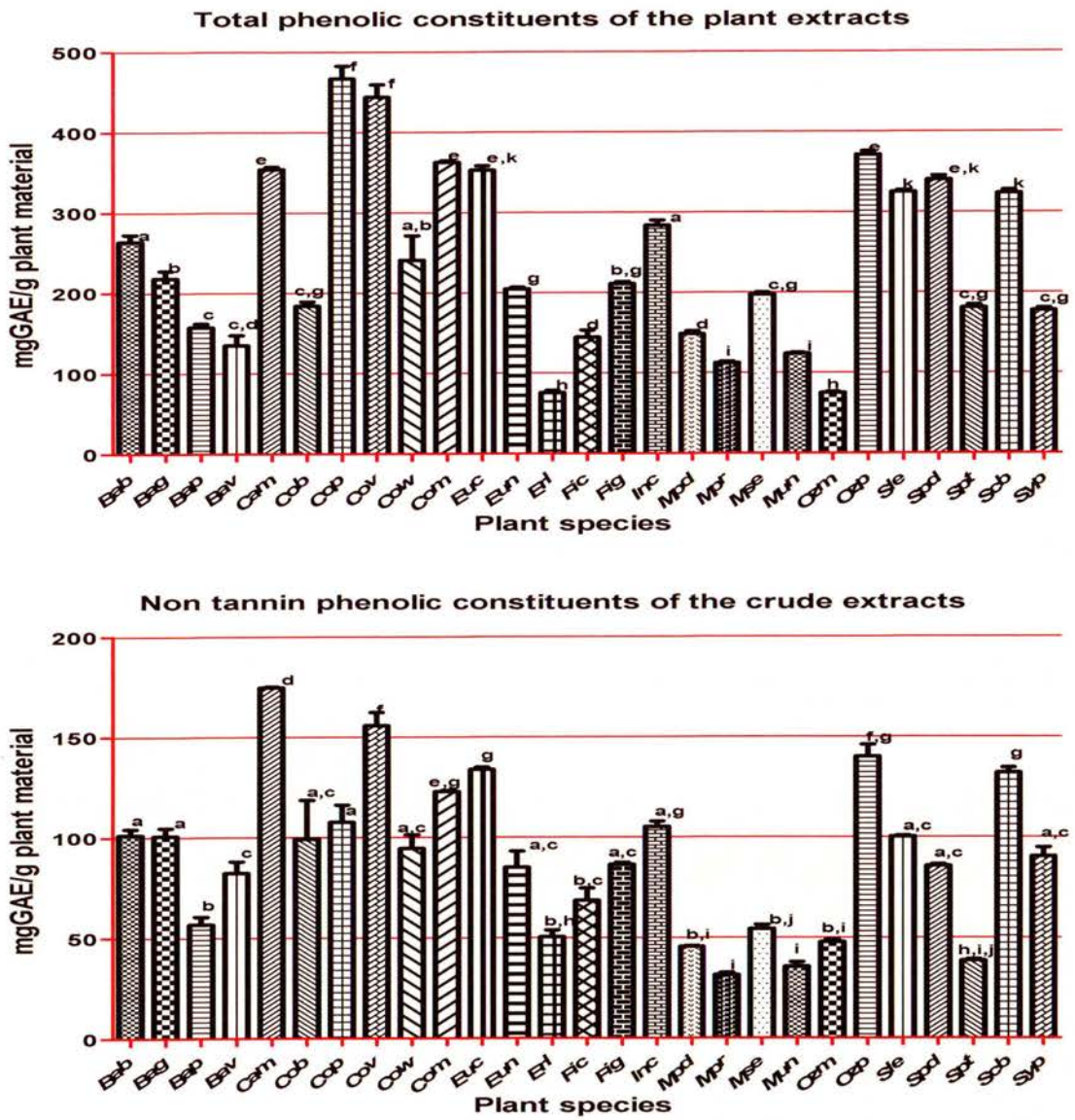

Fig. 3.6. Total phenolic and non-tannin constituent of the crude plant extracts

The plant with lowest content of non-tannin phenolic was *M. procumbens* (31.46±1.16 mg GAE/g plant material) which was not significantly different to *M. undata* (35.64±2.12 mg GAE/g plant material) $p< 0.05$.

The total tannin content of the extracts ranged from 25.55±0.81 to 359.40±8.30 mg GAE/g plant material (Fig.3.6). The highest tannin constituent was *C. padoides* (359.40±8.30 mg GAE/g plant material) and was mainly hydrolysable gallotannin (305.80±19.09 mg GAE/g plant material) (Fig 3.7). This was followed by *C. vendae* (288.40±8.30 mg GAE/g plant material) which also contained high hydrolysable gallotannin (197.60±12.79 mg GAE/g plant material)

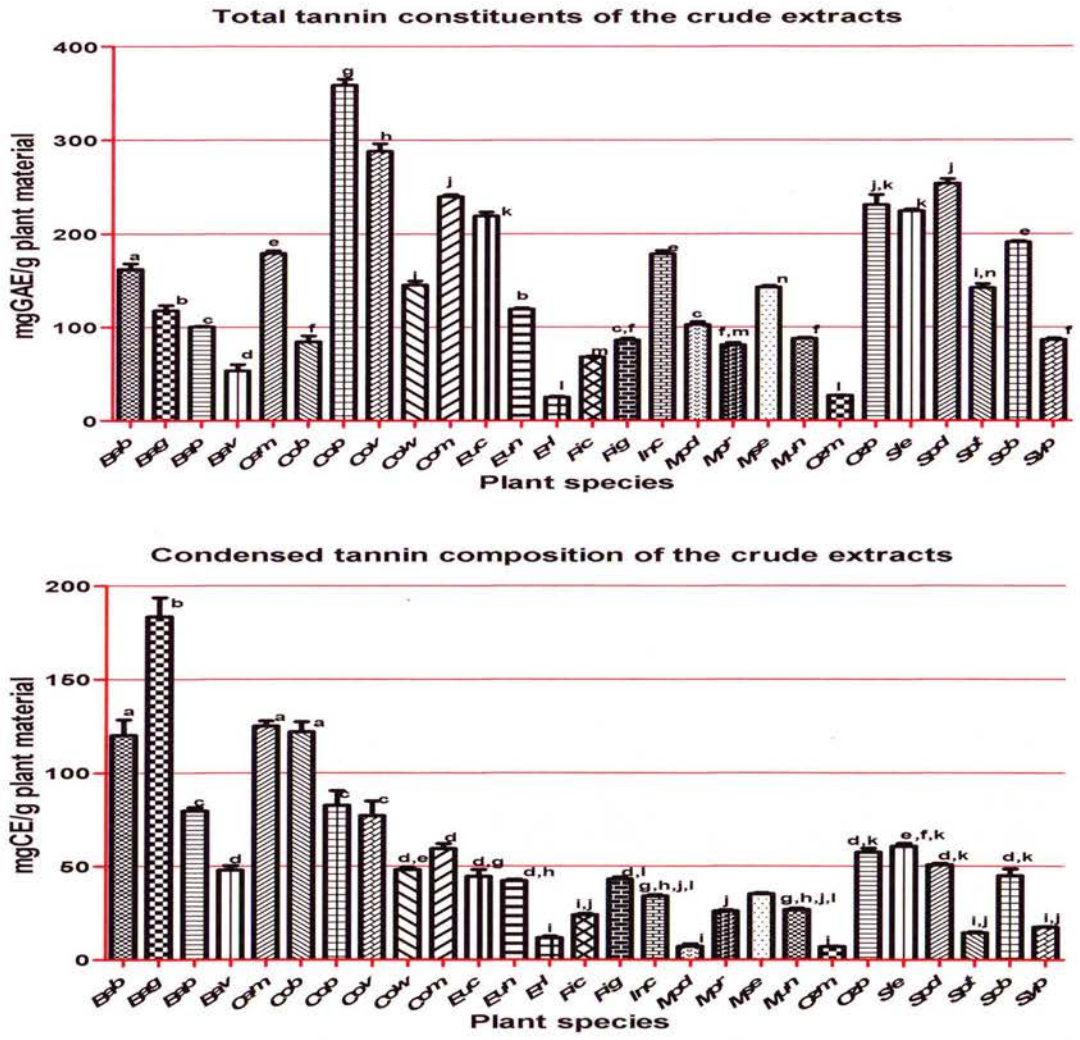

Fig. 3.7. Total tannin and condensed tannin of the crude extracts

The hydrolysable gallotannin constituents of *C. vendae* were not significantly different ($p< 0.05$) to that for *Euclea crispa* and *Indigofera cylindrical* at 199.36±17.61 and 185.21±11.50 mg GAE/g plant material respectively. *E. latissima* had the lowest tannin content at 25.55±0.81 mg GAE/g plant material followed by *O. mucronata* at

27.17±0.18 mg GAE/g plant material. For both these plants the tannin content was mainly proanthocyanidin at 33.42±3.76 and 19.88±2.51 mg LE/g plant material respectively (Fig 3.8).

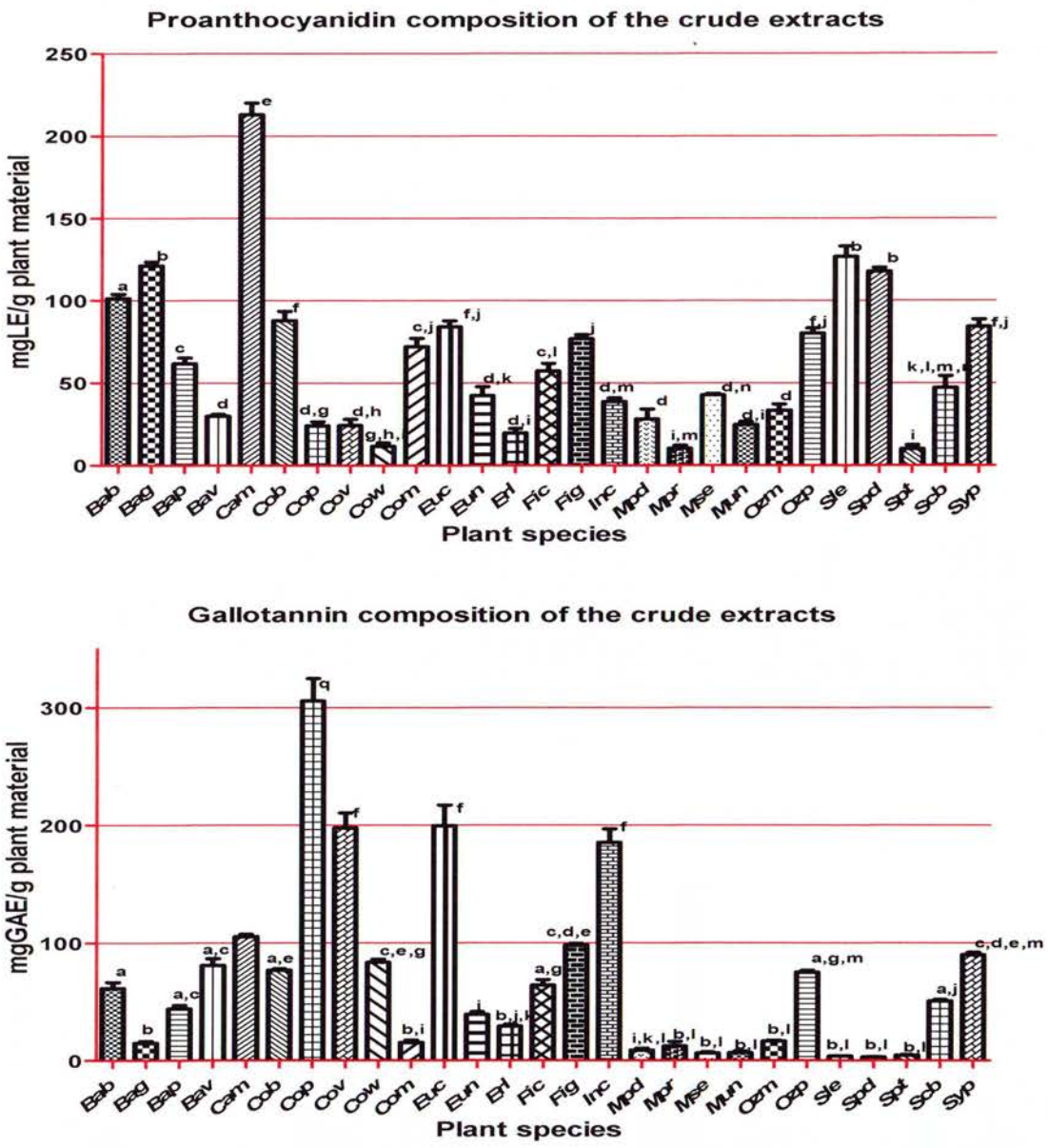

Fig. 3.8. Proanthocyanidin and gallotannin constituent of the crude extracts

The condensed tannin content ranged from 6.99±0.32 to 183.53±10.10 mg CE/g plant material. *Bauhinia galpinii* had the highest condensed tannin at 183.53±10.10 mg CE/g plant material. This was followed by *C. macrocarpa*, *Bauhinia bowkeri*, and *Combretum bracteosum* at 125.0±2.72, 120.02±8.37 and 121.90±5.50 mg CE/g plant material respectively, which are not significantly different ($p<0.05$) from each other. *O. mucronata* had the lowest

condensed tannin at 6.99±0.32 mg CE/g plant material followed by *M. pendulina* at 7.32±1.20 mg CE/g plant material and *E. latissima* at 11.90±0.8 mg CE/g plant material.

The highest proanthocyanidin content was found in *C. macrocarpa* at 213.10±7.00 mg LE/g plant material followed by *S. leptodictya*, *B. galpinii*, and *Searsia pendulina* at 126.54±6.46, 121.08±2.20 and 117.83±2.24 mg LE/g plant material respectively. Statistically, *S. leptodictya*, *B. galpinii*, and *Searsia pendulina* were not significantly different ($p<0.05$) for their proanthocyanidin content. *M. procumbens* and *S. pentheri* had the lowest proanthocyanidin content at 10.46±1.76 and 10.08±2.24 mg LE/g plant material respectively.

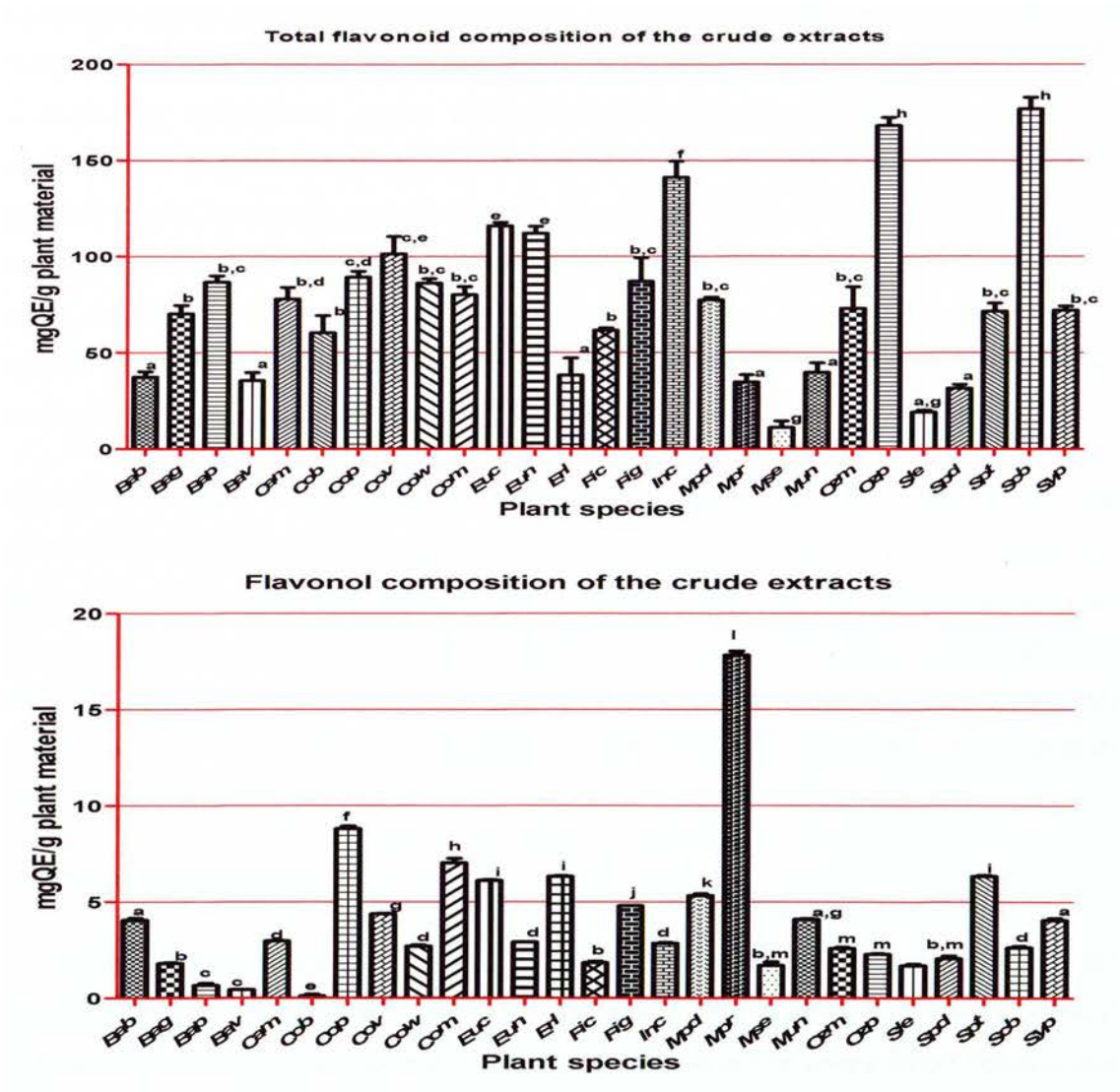

Fig. 3.9. Total flavonoids and the flavonol constituent of the crude extracts

The total flavonoid ranged from 11.27±3.37 to 176.87±5.96 mg QE/g plant material. The highest flavonoid content was present in *Schotia brachypetala* at 176.87±5.96 mg QE/g plant material followed by *O. paniculosa*

168.27±5.96 mg QE/g plant material. No significant difference ($p<0.05$) was present between the two plant (Fig 3.9). The lowest flavonoid content was present in *M. senegalensis* at 11.27±3.37 mg QE/g plant material. The highest flavonol content was present for *M. procumbens* at 17.85±0.20 mg QE/g plant material followed by *C. padoides* at 8.81±0.13 mg QE/g plant material. The lowest flavonol content was *C. bracteosum* at 0.13±0.07 mg QE/g plant material.

3.8. Discussion

3.8.1. Yield

The extraction of phenolic constituents from plant matrix is complex and is influenced by their chemical nature, extraction method, sample particle size, solvent as well as presence of interfering substances. Phenolics can also complex with carbohydrates, proteins, and other plant components like alkaloids. High molecular weight phenolics and their complexes are usually insoluble and solubility is also a function of the solvent polarity Naczk and Shahidi, 2004). Consequently, phenolic extracts composed of varied classes of phenolics present in different proportion with the degree of solubility in the solvent system as the primary determinant. In this experiment, simultaneous extraction and fractionation using acidified 70% acetone and n-hexane was adopted. Two immiscible phases of phenolic-enriched acetone solution (low phase) and chlorophyll-enriched terpenoids containing hexane (upper phase) were obtained.

The extraction process is an important factor for assessing the biological activity of medicinal plant extracts (Berlin and Berlin, 2005) as it influence yield of the extracts, extractive capacity of an extractant, and quality parameter of the herbal preparations (Albuquerque and Hanazaki, 2006). Low polar solvent extractants such as hexane, petroleum ether and dichloromethane extract non polar compounds mainly of terpenoids or highly methoxylated phenolics. In contrast, medium and high polar solvents such as ethyl acetate, acetone, methanol, ethanol water or mixture of these solvents extract the polar compounds ranging from simple phenolics to complex polymeric phenolics (tannins).

3.8.2. Thin layer chromatogram

TLC fingerprints of the plant leaf crude extracts and fractions showed complex mixtures of non-polar to polar compounds. TLC was used as qualitative method to characterize and document the phytochemical profiles of the extracts as a fingerprint. The phytochemical constituents of plants depend on several factors including seasonal changes, biotic (genetic) and abiotic (climatic stress, infection and soil fertility) factors (Moure *et al.*, 2001). TLC analyses help in monitoring composition of the extracts and fractions to ensure that no component(s) are lost during processing. It also provides a means of comparing phytochemical composition of different plant extracts developed side by side.

When comparing TLC fingerprints of the hexane fractions and the crude extracts from the extraction process, hexane fractions were enriched with non-polar components while the crude extracts were enriched with polar components, mostly of phenolic compounds. Solvents (2) E: F: A: W (70:5:15:10), (5) H: E: F (70:30:2), (8) C: E: F (50:40:10) and (9) E: M: W: F (50:6.5:5:2) were the best mobile phase obtainable for preparing TLC fingerprint of the crude extract, hexane fractions, dichloromethane fractions and ethyl acetate fractions respectively in this work.

Polyphenolic compounds are important bioactive component of medicinal plant extract exhibiting various pharmacological properties (Vundac *et al.*, 2007). Phenolic-enriched extracts have been reported to correlate with a wide range of physiological and health benefits which include antiallergenic, antiviral, antibacterial, antifungal (Pietta, 2000), antisecretory, antispasmolytic, antimotility (Yue *et al.*, 2004), anti-inflammatory, immunomodulatory and parasitic activities. In traditional medicine preparation of plant extract recipe, water or ethanolic solutions are the main extractants.

3.8.3. Phenolic constituents of the crude extracts

Polyphenolic compounds are important bioactive component of medicinal plant extract exhibiting various pharmacological properties (Vundac *et al.*, 2007). Phenolics form one of the main classes of secondary metabolites and several thousand (among them over 8,150 flavonoids) different compounds have been identified with a large range of structures: monomeric, dimeric and polymeric phenolics (Lattanzio *et al.*, 2006). Several classes of phenolics have been categorized on the basis of their basic skeleton. These groups of phytochemicals are primarily natural antioxidants which act as reducing agent, metal chelators and single oxygen quenchers. Phenolic-enriched extracts have been reported to correlate with a wide range of physiological and health benefits other than antioxidative activity.

Polyphenolic compounds have antidiarrhoea properties exhibiting one or more activities against diarrhoea pathogenesis. These may include antiallergenic, antiviral, antibacterial, antifungal (Pietta, 2000), antisecretory, antispasmolytic, antimotility (Yue *et al.*, 2004), anti-inflammatory, immunomodulatory and parasitic activities. In traditional medicine preparation of plant extract recipe, water or ethanolic solutions are the main extractants. These extractants extract more or less polar compounds made majorly of phenolic compounds. Specific types of phenolic compounds present in the crude extracts are therefore evaluated.

Flavonoids are C6-C3-C6 polyphenolic compounds present in food, beverage and medicinal plants. They have been reported to have useful pharmacological properties including anti-inflammatory activity, enzyme inhibitors, antiallergic, anti-inflammatory, antiviral, antispasmolytic, pro-secretory (Yue *et al.*, 2004) and antimicrobial activity. Flavonoids are known to act on the inflammatory response via many routes and block molecules like

COX, iNOS, cytokines, nuclear factor-κB and matrix metalloproteinases. In addition, flavonoids have good antioxidant, free radical scavengers that donate hydrogen, inhibit lipid peroxidation (Rauha, 2001; Havsteen, 2002) and metal ion chelators. However, the antioxidant power of flavonoids depends on some important structural prerequisites such as the number and the arrangement of hydroxyl groups, the extent of structural conjugation and the presence of electron-donating and electron-accepting substituents on the ring structure (Miliauskas *et al.*, 2005). These groups of phytochemicals are known to play some beneficial roles in the prevention of many oxidative and inflammatory diseases (Arts and Hollman, 2005) inhibiting oxidative and inflammatory enzymes (Middleton *et al.*, 2000).

Gallotannins are complex sugar esters of gallic acid and together with the related sugar esters of ellagic acid (ellagitannins) made up the hydrolysable tannins. Gallotannins exhibit biological activities including antimicrobial, antiviral, anti-inflammatory to anticancer and antiviral properties (Erde'lyi *et al.*, 2005). The mechanisms underlying the anti-inflammatory effect of tannins include the scavenging of radicals, and inhibition of the expression of inflammatory mediators, such as some cytokines, inducible nitric-oxide synthase, and cyclooxygenase-2 (Polya, 2003; Erde'lyi *et al.*, 2005).

Condensed tannins also referred to as proanthocyanidins are oligomers or polymers essentially derived from flavan-3-ol and their derivatives via carbon to carbon (C–C).or rarely C-O-C links. They differ structurally according to the number of hydroxyl groups on both aromatic rings (rind A and B) and the stereochemistry of the asymmetric carbons of the heterocyclic ring (ring C). Condensed tannins are classified according to their hydroxylation pattern into several subgroups including procyanidins (3,5,7,3',4'-OH), prodelphinidins, (3,5,7,3',4',5'-OH), propelargonidins (3,5,7,4'-OH), profisetinidins (3,7,3',4'-OH), prorobinetinidins (3,7,3',4',5'-OH), proguibourtinidins (3,7,4'-OH), proteracacinidins (3,7,8,4'-OH), and promelacacinidins (3,7,8, 3',4'-OH) (Cos *et al.*, 2003). As with other polyphenols, tannin structures are suitable for free radical scavenging activities serving as an excellent hydrogen or electron donors to form radicals that are relatively stable due to delocalization resulting from resonance and unavailability of site for attack by molecular oxygen (Mello *et al*, 2005). Tannins can also binds to some free radical producing enzymes forming an insoluble tannin-protein complex (astringent characteristic), complex with catalytic metallic ions making it unavailable to initiate oxidation reaction, and inhibiting lipid peroxidation process (Russo *et al*, 2005; Mello *et al*, 2005). These compounds are antagonists of hormone receptors or inhibitors of enzymes such as cyclooxygenase enzymes (Polya, 2003).

Tannins have the ability to protect renal renal cells against ischemia reperfusion injury (Yokozawa *et al*, 1997) characterized by an overproduction of $O_2^{\cdot-}$due to both an electron leak in the mitochondrial respiration chain and the conversion of xanthine dehydrogenase to xanthine oxidase (Wernes and Lucchesi, 1990). The protective action of tannins in this process is related to direct inhibition of enzymatic function of xanthine oxidase activity (Russo *et al*, 2005).

Production of reactive species (H_2O_2, $O_2^{\bullet-}$, and $OH^{\bullet}$) and peroxynitrite occurs at the site of inflammation and contributes to the exacerbation of inflammatory disease and tissue damage. In acute inflammation or chronic inflammations, the production of $O_2^{\bullet-}$ is increased at a rate that overwhelms the capacity of the endogenous SOD enzyme defence to dissipate. Reduction in the $O_2^{\bullet-}$ generation can decrease side-effects of the radical in inflammatory conditions. Tannins have been demonstrated to exhibit anti-inflammatory activity by exerting anti-oxidative properties in reducing $O_2^{\bullet-}$ and malondialdehyde (MDA) production, plasma extravasations and cell migration mainly of leukocytes and potentates the activity of SOD in radical scavenging (Nardi *et al*, 2007). It shows that reactive species are most important mediators that provokes or sustain inflammatory processes and consequently, their annihilation by antioxidants and radical scavenger can alleviate inflammation (Delaporte *et al*, 2002; Geronikaki and Garalas, 2006).

3.9. Conclusion

The extraction methods used optimally extract the phytochemical constituent from the powdered leaves. The extraction process adopted in this work separated the phytochemicals into non-polar hexane portion and polar water soluble portion in the first step. In addition to taxonomic identification and authentication of medicinal plant, chemical characterization is also an important and useful means of quality control as it directly correlate with pharmacological functions. The TLC fingerprints revealed the complexity of plant extracts and fractions with chemical compositions of a wide range of polarities. For the optimization of the TLC fingerprinting more than one mobile phase were used to obtain a representative chromatogram of the extracts. In this study, combination of fingerprint with multicomponent quantification of the phenolic compositions was adopted as a good method for chemical profiling of the plants.

There was a strong similarity in the chromatograms of *Erythrina latissima, Combretum vendae* and *Combretum woodii*. *Erythrina* and *Combretum* are not closely related and the similarity may be an example of convergent evolution. *Combretum vendae* and *C. woodii* are however, closely related as part of the subgenus *Combretum* and the results indicate the potential use of chemical markers in taxonomy.

CHAPTER FOUR

Antimicrobial activities of the plant extracts against potential diarrhoeal pathogens

4.0. Introduction

Infectious disease defined as an illness caused by a specific pathogen or its toxins that result from transmission of the causative agent or its virulence effectors from an infected person, animal or reservoir to a vulnerable host. The susceptibility of host to infectious pathogens, disease development, progression and severity depends on the age, gender, genetic, immune and nutritional status. Infectious diseases represent a leading cause of morbidity and mortality worldwide despite the advancement in orthodox medicine accounting for more than 26% of all death with developing countries carrying the major burden (Becker *et al.*, 2006). Infections are also considered to be a major contributing factor associated with reduced performance in food animals during growth. Particularly, persistent infections account for slow growth, suboptimal feeding efficiency and economic loss in the livestock industry (Borghetti *et al.*, 2009). The infective pathogens include bacteria, fungi, viruses, protozoa and parasites which manifest their virulence through different mechanisms (see section 2.2 for detailed discussion).

The discovery of antibiotics in 1928 and subsequent development in 1940 as medical treatment provides effective and efficient therapeutic agents for controlling almost all infectious diseases including many feared and contagious infections. Antibiotics are effective in curing many infectious diseases, but they also enhance selection of resistant microbes as some pathogens rapidly became resistant to many of the originally susceptible drugs (Barbour *et al*, 2004).

At present, the pharmaceutical drugs available to control antibiotic-resistant bacteria are becoming limited. The indiscriminate use and abuse of antibiotics has led to the development of antimicrobial resistance strains and toxicity of some drugs to human and animals (Barton, 2000; Parekh and Chanda, 2007). As a result of these problems, European Union (EU) with EU-directive 1831/2003 imposed ban on the use of antibiotics as growth factor in animal production with effect from 2006 to avoid cross resistance problem with human pathogens and chemical residues in foods (Makkar *et al*, 2007).

Drug resistance of human and animal pathogenic microbes and parasites has created a serious problem worldwide as previously treatable ailments such as diarrhoea (including dysentery and cholera), and tuberculosis are now more difficult and expensive to treat. The mechanisms of microbial resistance to antibiotic include (Dwyer *et al.*, 2009):

- Genetic alterations which involved the physical exchange of genetic material with another organism (via plasmid conjugation, phage-based transduction, or horizontal transformation), the activation of latent mobile genetic elements (transposons or cryptic genes), and the mutagenesis of its own DNA.

- Chromosomal mutagenesis arises directly from interaction between the chromosome and the antibacterial agent or antibiotic-induced oxidative stress, or indirectly from the bacterium's error prone DNA polymerases during the repair of a broad spectrum of DNA lesions.

The situations have complicated by the treatment of infectious diseases in immunocompromised patients. These negative health trends necessitate for a new prevention and treatment of infectious diseases including diarrhoea.

Medicinal plants have also featured as therapeutic agents used by the world population for basic health care needs and to combat many kinds of infectious diseases worldwide (Voravuthikunchai and Limsuwan, 2006). Medicinal plants have curative properties due to the presence of complex mixture of phytochemicals acting individually or synergistically to exert the associated therapeutic effects. Some of the plant compounds may be novel bioactive substances that can be effective as therapeutic agents for treating ailments such as infectious diarrhoea. These phytochemicals exhibit their antidiarrhoeal effects through various mechanisms such as antimicrobial (Lutherodt *et al*, 1999), increasing colonic water and electrolytic re-absorption, inhibition of intestinal motility (Oben *et al*, 2006) and anti-secretory effects (Rao *et al*, 1997). There is considerable research in the screening of natural products from extracts of edible and medicinal plants for the development of alternative drugs to prevent and curtail the emergence of drug-resistance pathogens or other forms of ailments.

4.1. Qualitative antimicrobial (Bioautography) assay

This refers to the direct bioactivity test on developed TLC plates as a means of localizing the biological activity such as microbial growth inhibition, enzymatic inhibition or antioxidative properties of extracts to the particular active compound(s). This helps in focusing attention on the relevant components of an extract (Saxena *et al.*, 1995) Fractionation of medicinal plant extracts in combination with bioautography provides an efficient and relative cheap method for bioactivity-guided isolation of target compound(s) (Hostettman *et al.*, 1997). Practical application of bioautography in activity guided isolation includes enzyme inhibition assay such as the Ellman method for cholinesterase inhibitors (Ellman *et al.*, 1961). In this method, the developed TLC plate is sprayed with a substrate, enzyme and indicator to determine the inhibition by colour variation (white zone against yellow background) (Rhee *et al.*, 2001).

In the antimicrobial bioassay, two bioautography methods are available. Firstly, the agar diffusion method involves pouring a layer of inoculated agar solution of the microbes on the developed TLC plate and allowed to set, and the bioactive zone(s) are transferred to the agar gel by diffusion where they can inhibit the growth of the microorganism (Fig 4.1). Secondly, the direct method involves spraying of microorganism broth inoculant onto the TLC plate (Homans and Fuchs, 1970) and incubating in humid conditions to facilitate the growth of the organism. Microbial growth inhibitions are recognized based on the ability of the living microorganism to

transform tetrazolium salts to a coloured formazan product. White spots against an intense purple coloured background indicate the compound(s) that kill the tested microorganism (Hostettman and Martson, 2002).

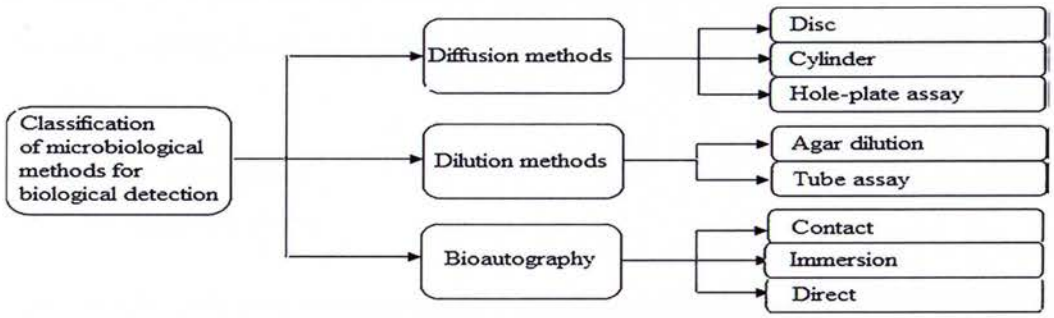

Fig 4.1. The classification of microbiological methods for biological detection (Adopted from Choma and Grzelak, 2011)

4.2. Quantitative antimicrobial activity (Minimum inhibitory concentration (MIC)) assay

Among the quantitative antimicrobial methods used in evaluating plant extract activity, agar diffusion assay (Greenwood, 1989) and two-fold serial micro-dilution assay (Eloff, 1998) are the most common in phytomedicine research. Sensitivity of the two protocols and their mechanisms varied widely. The mechanism of agar diffusion is the movement of bioactive compounds through the solid agar medium to kill or inhibit the growth of organism it may come in contact with. However, agar diffusion assays may sometimes lead to a false negative result, due to influence of the agar type, salt concentration, incubation temperature molecular size of the antimicrobial components (Greenwood, 1989), and limited diffusion of bioactive component in agar medium. The two-fold serial micro-dilution assay depend on direct contact between the test sample and organism is adjudged to be 30 times more sensitive than the other methods used to screen plant extracts for antimicrobial activity (Eloff, 1998). Although, the effective solubility and miscibility of the bioactive component in the test medium such as the non-polar compounds like terpenes, alkaloid and highly methoxylated phenolics is a limiting factor.

4.3. Selection of microorganisms used in the study

The selection of the microorganisms for antibacterial evaluation in this study was based on their known pathogenic effects in both human and animals with emphasis on diarrhoeal pathogens. Pathogenic *E. coli* has been implicated in diseases such as diarrhoea, haemorrhagic colitis, haemolytic uremic syndrome and thrombocytopenic purpura (Voravuthikunchai and Limsuwan, 2006). *Enterococcus faecalis* has been implicated in causing enteric infection with diarrhoeal effects (Butler, 2006). *Pseudomonas aeruginosa* strains cause diseases such as mastitis, abortions and upper respiratory complications (Masika and Afolayan, 2002). *Staphylococcus aureus* is one of the prominent microbes causing skin infection such as boils, abscesses, carbuncles and sepsis of wounds and it also produces toxins causing diarrhoea and vomiting (Maregesi *et al*,

2008). *Candida albicans* is a typical opportunistic pathogen causing diarrhoea (Gambhir *et al*, 2006), oral and vaginal candidiasis (Shai *et al*, 2008) especially in immunocompromised individuals due to unexpected opportunity by a failure of host defence. *Cryptococcus neoformans* has been implicated in causing life-threatening meningoencephalitis (Xue *et al*, 2007) and pneumonia in immunocompromised individuals (Hamza *et al*, 2006).

4.4. Material and Methods

4.4.1. Microorganism strains

Two standard strains of Gram-positive bacteria (*Staphylococcus aureus* ATCC 29213, *Pseudomonas aeruginosa* ATCC 25922) and two standard strains of Gram-negative bacteria (*Escherichia coli* ATCC 27853, *Enterococcus faecalis* ATCC 29212) were used for antibacterial assay. Three clinical pathogenic fungi namely yeasts (*Candida albicans, Cryptococcus neoformans*) and mould (*Aspergillus fumigatus*) (All fungal strains obtained from the Department of Veterinary Tropical Diseases, Faculty of Veterinary Sciences, University of Pretoria) were used.

4.4.2. Culturing of the Bacteria

The bacterial strains were maintained in Mueller Hinton agar (MHA) (Fluka, Spain) while the fungi were maintained in Sabouraud dextrose agar (Merck, Germany) at 4°C under anaerobic conditions. All the organisms were subcultured every 2 weeks. Before testing, the bacterial inoculums were prepared and cultivated in Mueller Hinton broth for 12 h at incubation temperature of 37°C. The fungi inoculums were prepared in Sabouraud dextrose broth (SDB,). The microbial cultures were serially diluted (10 fold increments) in sterile broth to obtain the cell suspension of 1.0×10^5 CFU/ml.

4.4.3. Bioautography against some pathogenic microorganisms

Bioautography was undertaken to ascertain the number of active compound(s) present in crude extracts and fractions. TLC plates were developed as described in section 3.5.4 (Pp 43 - 44), and sprayed with overnight cultures of *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli, Enterococcus faecalis*, *Candida albicans, Cryptococcus neoforman*) and *Aspergillus fumigatus* in a biosafety class II fume carbinet (Labotec South Africa. The sprayed chromatograms were incubated at 37°C for 12-16 h before spraying with tetrazolium violet (INT). The inhibitory activity of any components was evident as clear white zones against the purple/red background.

4.4.4. Determination of Minimum Inhibitory Concentration (MIC) against the bacteria pathogens

The minimum inhibitory concentration (MIC) for the crude extract and fractions against bacteria were evaluated using the twofold serial dilution assay with tetrazolium violet added as growth indicator (Eloff, 1998). The extracts

(100 µl) at an initial concentration of 1.0 × 10^4 µg/ml was serially diluted with distilled water up to 50% in 96-well microtitre plate to prepare solution range between 5000 µg/ml first well and 40 µg/ml last well. The bacterial (100 µl) inoculants from 12 h broth cultures (section 4.4.2) diluted to 1:100 were added to each well to obtain final extract concentration range of 2500 µg/ml first well and 20 µg/ml last well. Gentamicin (25 µg/ml first well and 0.18 µg/ml last well) was used as positive control and the solvent used in dissolving the extract was used as negative control. Final volume in each well was 200 µl. The plates were incubated for 24 h at 37°C and 100% relative humidity. The inhibition of the bacteria were visualised by adding 40 µl of aqueous p-iodonitrotetrazolium violet (INT) (Sigma) to each well (concentration 200 µg/ml). The plates were incubated for another 1 h and MIC was determined as the lowest concentrations of test sample before purple formazan colour were observed.

4.4.5. Determination of Minimum Inhibitory Concentration (MIC) against the fungal pathogens

Minimum inhibitory concentrations (MIC) against three pathogenic fungi were determined using twofold serial dilution assay as described above by Eloff, 1998 with the following modification of Masoko *et al*, 2005. The fungal inoculants (100 µl) were in fresh Sabouraud dextrose broth and positive controls was amphotericin B (50 µg/ml first well and 0.4 µg/ml last well) and negative controls was 70% acetone, final visualization of inhibitory activity was obtained after an incubation for 24 h at 37°C, and 100% relative humidity.

4.5. Results

4.5.1. Microbial bioautography

The TLC bioautography of the crude extracts and fractions of the 27 plant species tested against standard strain bacteria and clinical fungal isolates are presented in Fig 4.2-4.12.

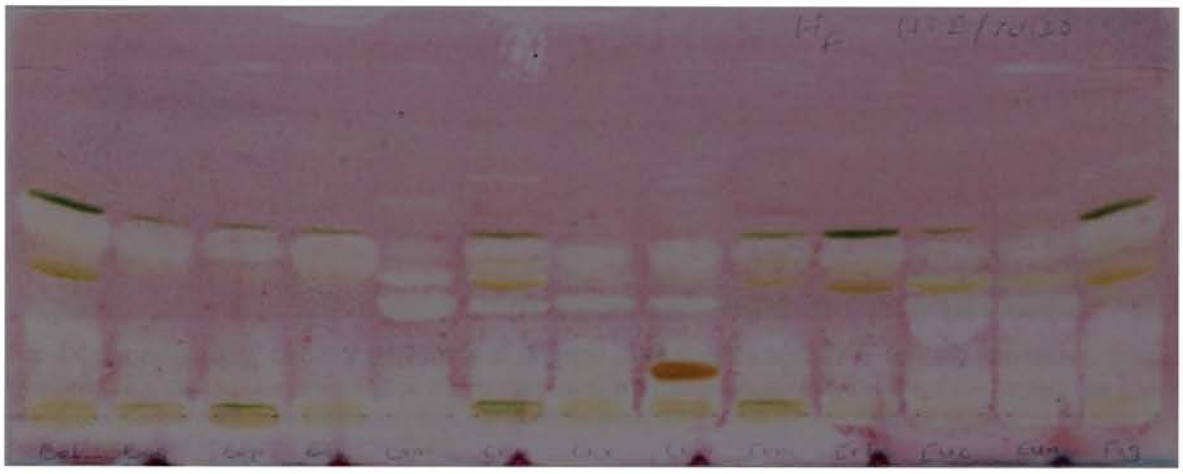

Fig. 4.2. Bioautography of hexane (upper) fraction of different plant species against *S. aureus* (Bab (*Bauhinia bowkeri*), Bag (*Bauhinia galpinii*), Bap (*Bauhinia petersiana*),Cam (*Carissa macrocarpa*), , Cop (*Combretum padoides*), Cov (*Combretum vendae*), Cow (*Combretum woodii*), Com (*Commiphora harveyi*), Euc (*Euclea crispa*), Eun (*Euclea natalensis*), Erl (*Erythrina latissima*), Fic (*Ficus craterestoma*), Fig (*Ficus glumosa*) developed with hexane: ethyl acetae: formic acid (70:30))

The antimicrobial activities of the extracts were concentrated on the non-polar-enriched hexane fraction while the polar enriched components no sign of microbial inhibition.

Fig. 4.3. Bioautography of dichloromethane fractions of different plant species against *S. aureus* (Bab (*Bauhinia bowkeri*), Bag (*Bauhinia galpinii*), Bap (*Bauhinia petersiana*),Bav (*Bauhinia variegata*) Cam (*Carissa macrocarpa*), , Cop (*Combretum padoides*), Cov (*Combretum vendae*), Cow (*Combretum woodii*), Com (*Commiphora harveyi*), Euc (*Euclea crispa*), Eun (*Euclea natalensis*), Erl (*Erythrina latissima*), Fic (*Ficus craterestoma*), Fig (*Ficus glumosa*) developed with chloroform: ethylacetate: formic acid (100:13:10)).

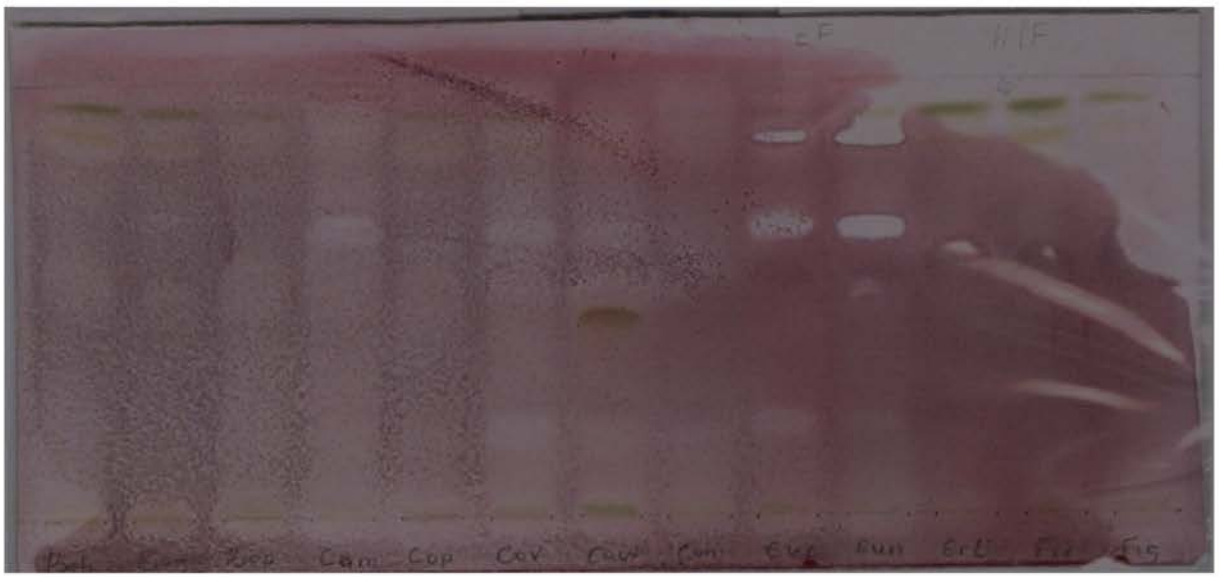

Fig. 4.4. Bioautography of hexane fractions of different plant species against *E. faecalis* (Bab (*Bauhinia bowkeri*), Bag (*Bauhinia galpinii*), Bap (*Bauhinia petersiana*),Cam (*Carissa macrocarpa*), Cop (*Combretum padoides*), Cov (*Combretum vendae*), Cow (*Combretum woodii*), Com (*Commiphora harveyi*), Euc (*Euclea crispa*), Eun (*Euclea natalensis*), Erl (*Erythrina latissima*), Fic (*Ficus craterestoma*), Fig (*Ficus glumosa*) developed with hexane: ethyl acetae: formic acid (70:30))

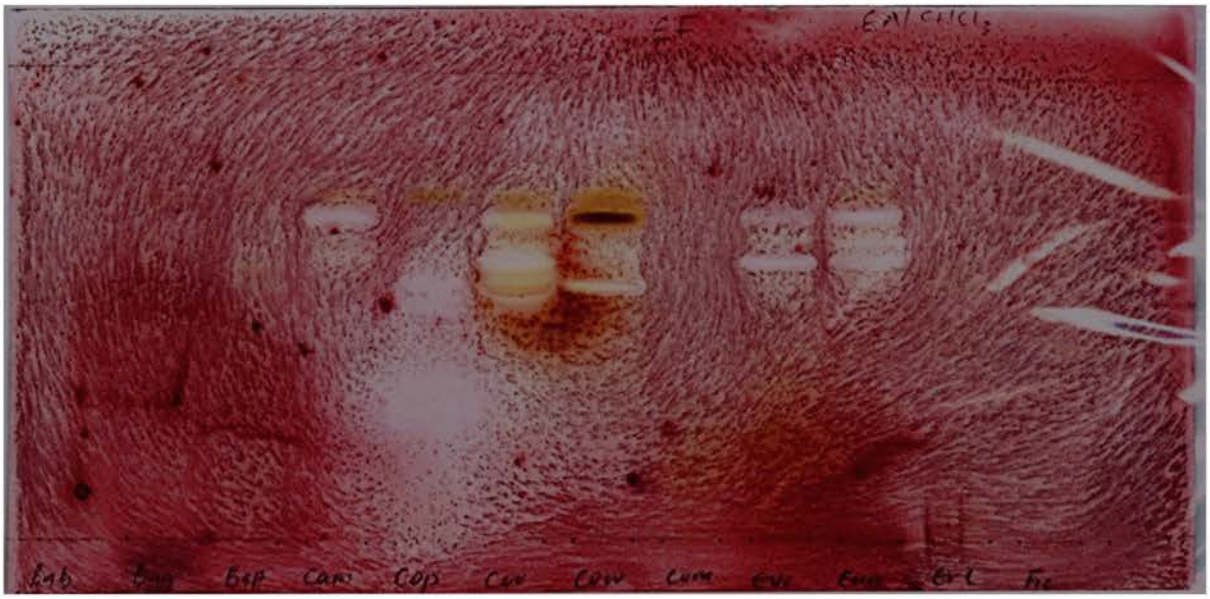

Fig. 4.5. Bioautography of dichloromethane fractions of different plant species against *E. coli* (Bab (*Bauhinia bowkeri*), Bag (*Bauhinia galpinii*), Bap (*Bauhinia petersiana*),Bav (*Bauhinia variegata*) Cam (*Carissa macrocarpa*), , Cop (*Combretum padoides*), Cov (*Combretum vendae*), Cow (*Combretum woodii*), Com (*Commiphora harveyi*), Euc (*Euclea crispa*), Eun (*Euclea natalensis*), Erl (*Erythrina latissima*), Fic (*Ficus craterestoma*) developed with chloroform: ethylacetate: formic acid (100:13:10)).

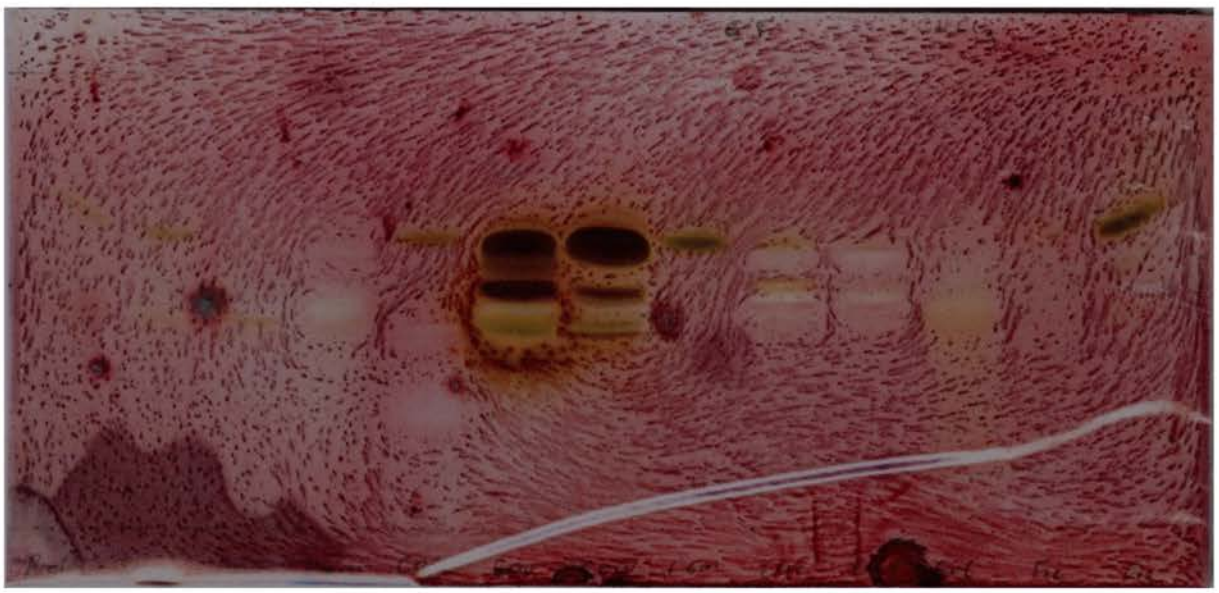

Fig. 4.6. Bioautography of dichloromethane fractions of different plant species against *E. faecalis* (Bab (*Bauhinia bowkeri*), Bag (*Bauhinia galpinii*), Bap (*Bauhinia petersiana*),Bav (*Bauhinia variegata*) Cam (*Carissa macrocarpa*), , Cop (*Combretum padoides*), Cov (*Combretum vendae*), Cow (*Combretum woodii*), Com (*Commiphora harveyi*), Euc (*Euclea crispa*), Eun (*Euclea natalensis*), Erl (*Erythrina latissima*), Fic (*Ficus craterestoma*), Fig (*Ficus glumosa*) developed with chloroform: ethylacetate: formic acid (100:13:10)).

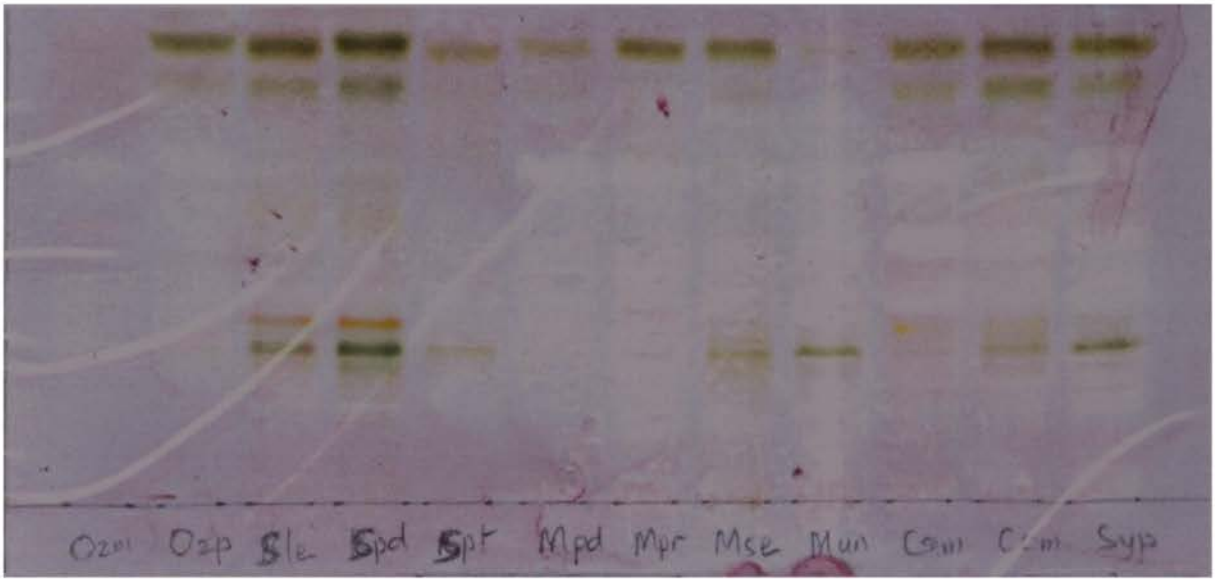

Fig. 4.7. Bioautography of hexane of different plant speicies against *C. neoformans* (Ozm (*Ozoroa mucronata*), Ozp (*Ozoroa paniculosa*), Sle (*Searsia leptodictya*), Spd (*Searsia pendulina*), Spt (*Searsia pentheri*), Mpd (*Maytenus peduncularis*), Mpr (*Maytenus procumbens*), Mse (*Maytenus senegalensis*), Mun (*Maytenus undata*), Cam (*Carissa macrocarpa*), Com (*Commiphora harveyi*), Syp (*Syzygium paniculatum*)).

Fig. 4.8. Bioautography of dichloromethane fractions against *C. neoformans* (Ozm (*Ozoroa mucronata*), Ozp (*Ozoroa paniculosa*), Sle (*Searsia leptodictya*), Spd (*Searsia pendulina*), Spt (*Searsia pentheri*), Mpd (*Maytenus peduncularis*), Mpr (*Maytenus procumbens*), Mse (*Maytenus senegalensis*), Mun (*Maytenus undata*), Cam (*Carissa macrocarpa*), Com (*Commiphora harveyi*), Syp (*Syzygium paniculatum*))

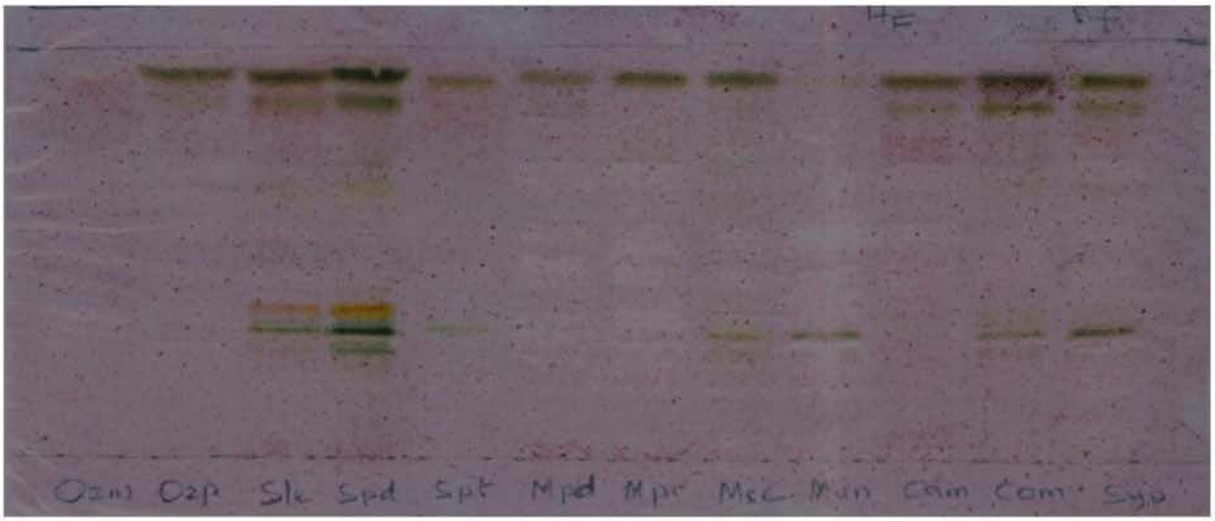

Fig. 4.9. Bioautography of hexane fractions against *A. fumigatus* (Ozm (*Ozoroa mucronata*), Ozp (*Ozoroa paniculosa*), Sle (*Searsia leptodictya*), Spd (*Searsia pendulina*), Spt (*Searsia pentheri*), Mpd (*Maytenus peduncularis*), Mpr (*Maytenus procumbens*), Mse (*Maytenus senegalensis*), Mun (*Maytenus undata*), Cam (*Carissa macrocarpa*), Com (*Commiphora harveyi*), Syp (*Syzygium paniculatum*))

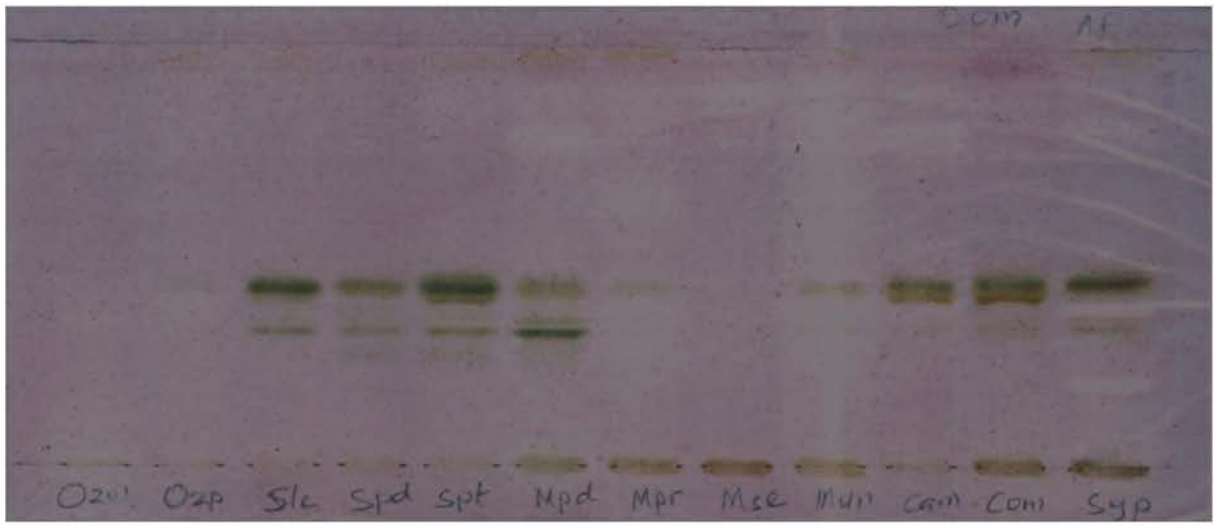

Fig. 4.10. Bioautography of dichloromethane fractions against *A. fumigatus* (Ozm (*Ozoroa mucronata*), Ozp (*Ozoroa paniculosa*), Sle (*Searsia leptodictya*), Spd (*Searsia pendulina*), Spt (*Searsia pentheri*), Mpd (*Maytenus peduncularis*), Mpr (*Maytenus procumbens*), Mse (*Maytenus senegalensis*), Mun (*Maytenus undata*), Cam (*Carissa macrocarpa*), Com (*Commiphora harveyi*), Syp (*Syzygium paniculatum*))

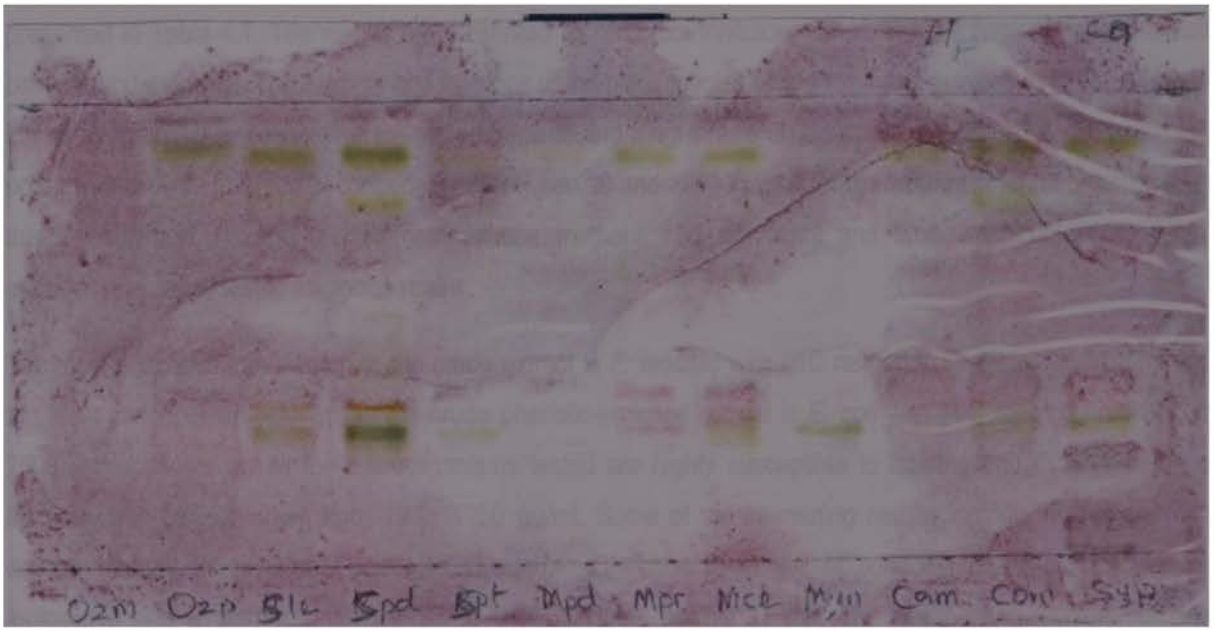

Fig. 4.11. Bioautography of hexane fractions against *C. albicans* (Ozm (*Ozoroa mucronata*), Ozp (*Ozoroa paniculosa*), Sle (*Searsia leptodictya*), Spd (*Searsia pendulina*), Spt (*Searsia pentheri*), Mpd (*Maytenus peduncularis*), Mpr (*Maytenus procumbens*), Mse (*Maytenus senegalensis*), Mun (*Maytenus undata*), Cam (*Carissa macrocarpa*), Com (*Commiphora harveyi*), Syp (*Syzygium paniculatum*)).

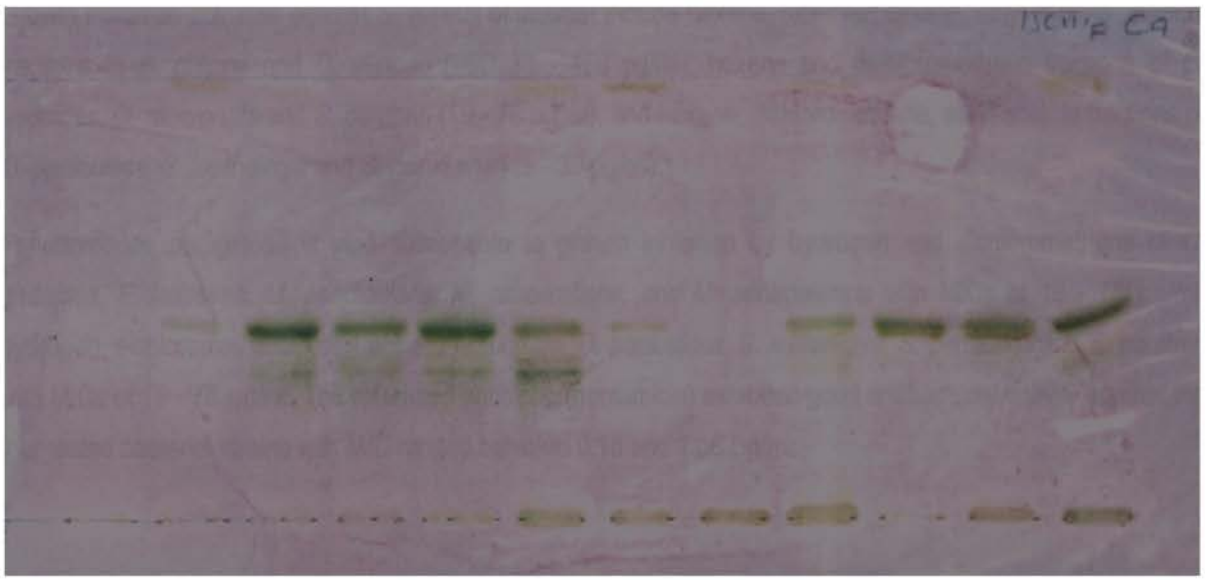

Fig. 4.12. Bioautography of dichloromethane fractions against *C. albicans* (Ozm (*Ozoroa mucronata*), Ozp (*Ozoroa paniculosa*), Sle (*Searsia leptodictya*), Spd (*Searsia pendulina*), Spt (*Searsia pentheri*), Mpd (*Maytenus peduncularis*), Mpr (*Maytenus procumbens*), Mse (*Maytenus senegalensis*), Mun (*Maytenus undata*), Cam (*Carissa macrocarpa*), Com (*Commiphora harveyi*), Syp (*Syzygium paniculatum*))

4.5.2. Minimum inhibitory concentration (MIC)

The antibacterial activities of the phenolic-enriched extracts, fractions and sub-fractions of different polarities of 27 plant species evaluated against 4 microorganisms (two gram positive and two gram negative bacteria) are

presented in Table 4.1. The results are presented as minimum inhibitory concentrations (µg/ml) against tested bacteria and the fungi. The extracts and fractions of the plant species tested exhibited average to good degree of inhibition against the growth of all the tested bacteria and fungi strains. The extracts and fractions exhibited high potency or growth inhibition at concentration between 19 and >2500 µg/ml for the various different organisms. In this investigation, hexane and dichloromethane fractions had significant and broad-spectrum antimicrobial activities against all tested microbial strains.

The most susceptible bacterium to the crude extract is *E. faecalis* with MIC ranged from 78 to 1250 µg/ml and the least susceptible bacterium to the crude phenolic-enriched extract is *E. coli* with MIC ranging from 312 to 2500 µg/ml. However, all the microorganisms tested are highly susceptible to hexane and dichloromethane fractions with MICs ranging from 19 to 1250 µg/ml. Some of the interesting results include the hexane and dichloromethane fractions of *C. padoides, C. vendae, C. woodii, B. galpinii, M. penducularis, M. procumbens, S. leptodictya* and *S. pendulina* with MICs of 19 - 39 µg/ml against *E. coli*. The hexane and dichloromethane fractions of *C. padoides, C. vendae, C. woodii, M. penducularis, M. procumbens, M. senegalensis, O. mucronata, O. paniculosa, S. leptodictya* and *S. pentheri* also exhibited good microbial growth inhibitory activity against *E. faecalis* with MICs between 19 - 78 µg/ml.

Growth inhibition activities against *S. aureus* of interest include hexane, dichloromethane, ethyl acetate, butanol fractions of *B. galpinii* and *C. vendae* (MIC 39 - 156 µg/ml); hexane and dichloromethane fractions of *C. padoides*, *O. mucronata* and *S. pentheri* (19 - 78 µg/ml), and hexane, dichloromethane, ethyl acetate fractions of *O. paniculosa, S. leptodictya* and *S. pendulina* (19 - 39 µg/ml).

Pseudomonas aeruginosa is also susceptible to growth inhibition by hydrogen and dichloromethane of *C. padoides, E. latissima, M. pendicularis, M. procumbens, and M. senegalensis* with MICs of 19 - 78 µg/ml; hydrogen, dichloromethane, ethyl acetate *M. undata, O. paniculosa, S. leptodictya, S. pendulina* and *S. pentheri* with MICs of 19 - 78 µg/ml. The reference antibiotic (gentamicin) exhibited good antibacterial activity against the four tested bacterial strains with MIC ranged between 0.18 and 1.56 µg/ml.

Table 4.1. Minimum inhibitory concentration (MIC) of the crude extracts and fraction against *E. coli* and *E. faecalis*

	E. coli						*E. faecalis*					
Plant spp	CRE	Hf	DCMf	ETOAc	Butanol	Water	CRE	Hf	DCMf	ETO Ac	Butanol	Water
Bab	625	39	156	312	156	>2500	78	312	312	39	78	312
Bag	312	39	156	312	156	312	156	156	39	156	78	312
Bap	312	78	156	312	625	312	78	156	312	78	39	312
Bav	625	78	156	156	156	312	312	312	625	39	39	312
Cam	312	156	156	156	312		312	312	312	78	156	
Cob	312	39	156	312	156	>2500	156	312	312	39	78	312
Cop	156	19	19	312	39	>2500	78	19	19	156	78	312
Cov	312	39	39	156	156	312	156	39	78	78	78	39
Cow	625	39	39	312	156	>2500	156	39	78	78	78	312
Cmh	625	312	39	78	312		156	156	156	78	156	
Erl	1250	156	19	156	156	1250	156	78	19	78	78	1250
Euc	312	625	78	78	156		156	312	156	156	78	
Eun	312	312	312	78	156		625	312	312	156	156	
Fic	2500	312	78	312	312		2500	78	312	312	312	
Fig	1250			1250	312		312			625	156	
Inc	1250				312		1250				312	
Mpd	312	39	39	78	625			39	39	156	1250	
Mpr	1250	39	39	156	1250		>2500	39	39	156	625	
Mse	2500	78	78	156	1250		2500	39	39	312	625	
Mun	1250	312	156	156	>2500		312	156	156	312	2500	
Ozm	1250	78	39	156	312		625	39	19	625	1250	
Ozp	1250	78	39	39	625		1250	39	19	156	78	
Sle	1250	39	39	78	2500		625	39	19	156	625	
Spd	1250	39	39	78	1250		625	156	19	312	625	
Spt	2500	39	39	156	2500		1250	39	19	156	1250	
Scb	625	312	156	39	156	1250	1250	78	312	39	78	2500
Syp	625	312	312	39	156	312	1250	312	156	39	156	1250

Table 4.1. Cont.....Minimum inhibitory concentration (MIC) of the crude extracts and fraction against *S. aureus* and *P. aeruginosa*

	S. aureus						*P. aeruginosa*					
Plant spp	CRE	Hf	DCMf	ETOAc	Butanol	Water	CRE	Hf	DCMf	ETOAc	Butanol	Water
Bab	625	78	312	625	78	312	312	78	312	156	312	312
Bag	625	39	78	39	78	312	312	312	78	312	156	312
Bap	312	156	312	78	78	78	78	312	625	625	312	625
Bav	625	78	1250	-	-	-	156	78	625	625	312	625
Cam	312	312	156	156	78		312	156	625	312	78	
Cob	625	78	312	156	78	312	312	78	312	625	156	312
Cop	156	19	39	156	156	312	156	78	39	312	19	312
Cov	156	39	78	78	39	39	156	156	156	156	39	312
Cow	312	39	78	156	156	312	625	312	156	312	312	312
Cmh	312	78	156	78	156	2500	1250	312	312	156	312	
Erl	1250	78	312	78	312		2500	78	78	156	78	1250
Euc	156	312	312	78	312		156	312	625	156	39	
Eun	312	312	625	312	312		625	625	625	312	78	
Fic	2500	156	312	1250	1250		2500	156	312	625	156	
Fig	625			312	625		1250			312	78	
Inc	625				156		625				312	
Mpd	>2500	78	39	312	78		312	19	19	156	312	
Mpr	1250	39	78	312	625		312	78	39	156	625	
Mse	625	78	39	156	625		625	39	39	156	1250	
Mun	625	156	156	156	312		312	78	78	78	2500	
Ozm	2500	19	19	625	1250		312	156	39	312	2500	
Ozp	156	19	19	78	156		156	39	39	78	156	
Sle	312	19	19	78	312		625	39	39	39	625	
Spd	312	78	78	39	625		625	78	78	19	2500	
Spt	312	19	19	156	312		156	19	19	78	2500	
Scb	156	1250	156	156	156	1250	156	312	156	156	156	1250
Syp	156	39	156	312	312	2500	156	156	156	78	312	1250

4.5.3. Minimum inhibitory concentration (MIC)

The phenolic-enriched crude extracts and fraction exhibited good to moderate growth inhibitory activities against the three fungal strains of different morphology with MICs ranging from 19 to 2500 µg/ml (Table 4.2). *Candida albicans* demonstrated resistance to all the crude extracts and fractions with the exception of dichloromethane and butanol fractions which had MICs of 19 - 78 µg/ml. In contrast, Cryptococcus neoformans was sensitive to majority of many crude extracts and fractions at the concentration ranging from 19 - 78 µg/ml. The fungi were susceptible to amphotericin B with the MIC ranges from 0.78 - 6.25 µg/ml.

Table 4.2. Minimum inhibitory concentration (MIC) of the crude extracts and fraction against *C. albicans*, *C. neoformans* and *A. fumigatus* Values below 100 µg/ml in different colour

	C. albicans						*C. neoformans*						*A. fumigatus*					
Plant spp	CRE	H	DCM	ETOAc	But	Water	CRE	H	DCM	ETOAc	But	Water	CRE	H	DCM	ETOAc	But	Water
Bab	625	625	78	156	39	39	156	312	78	78	39	39	2500	156	312	312	156	625
Bag	312	625	78	156	78	625	78	315	78	39	39	312	625	156	156	156	78	156
Bap	1250	156	78	156	156	625	78	625	39	39	156	78	625	312	78	78	156	156
Bav	312	-	-	156	78	625	78	-	-	78	78	78	625			312	156	156
Cam	625	156	625	312	19	156	78	78	156	19	19	1250	625	19	1250	625	312	156
Cob	625	625	312	156	156	1250	78	625	625	39	19	39	312	312	156	156	312	625
Cop	312	312	19	312	156	625	39	156	625	19	312	312	156	156	78	78	156	625
Cov	1250	625	312	78	312	312	78	19	156	78	156	312	156	156	156	156	156	625
Cow	1250	625	19	156	78	625	78	312	312	39	156	312	156	156	78	78	78	625
Cmh	625	156	39	156	39	625	156	39	19	39	19	1250	625	39	19	1250		312
Erl	1250	156	156			625	312	78	19			625	1250	156	156	312		625
Euc	625	156	156	156	78	156	78	156	78	39	39	156	312	625	156	625	156	156
Eun	625	156	78	78	78	625	78	78	39	156	78	625	312	625	625	312	78	312
Fic	2500	78	39	156	39	312	312	78	19	156	39	625	1250	312	156	156	312	156
Fig	625	78	39	78	39	312	78	78	19	78	39	1250	625	78	78	78	625	312
Inc	1250	78	19	156			78	78	39	39			1250	78	156			
Mpd	312	312	78	312	156	312	78	312	78	78	1250	2500	312	78	156	156	312	625
Mpr	1250	156	39		312	625	625	156	39		156	2500	1250	78	78		156	625
Mse	1250	78	39	156	156	625	156	156	39	78	78	1250	625	156	78	156	1250	312
Mun	1250	625	78	312	625	1250	156	1250	78	78	312		1250	625	625	156	625	625
Ozm	625	312	156	156	625	625	156	312	156	39	156	2500	625	39	156	39	156	1250
Ozp	312	156	78	156	312	625	78	312	39	39	156	1250	312	78	78	312	156	625
Sle	312	39	39		312	625	156	156	39		312	1250	625	78	39		156	312
Spd	625	39	78	156	625	625	312	156	39	39	156	625	625	78	39	156	78	312
Spt	2500	625	78	312	156	1250	312	1250	19	39	312	1250	2500	78	19	625	156	625
Scb	625	156	312			625	78	312	78			2500	312	78	19			625
Syp	312	156	156	625	312	625	156	78	39	78	39	1250	312	156	19	1250	2500	312

4.6. Discussion

4.6.1. Antimicrobial bioautography

The crude extracts and various fractions were screened qualitatively for growth inhibitory activity against 4 bacteria and 3 fungi representing different morphologies as yeasts and moulds. Many compounds present in the non-polar enriched hexane fractions inhibited the growth of the organisms tested with several zones of inhibitions.

However, the polar fractions exhibited poor individual inhibitory activities, and this may be due to the high solubility of polar compounds in water, like flavonoids resulting in washing and spread of the compounds on the TLC plate surface. Therefore, reducing the threshold inhibitory concentrations of the bioactive compounds on the spot of the chromatogram against the organisms tested. Other factors may include the disruption of synergistic effects of the individual compounds separated on TLC plates or the concentration of the bioactive components is not sufficient to inhibit microbial growth.

A number of methods have been developed for effective and quick screening of microbial growth inhibitory properties of compounds like the disc or agar diffusion assay adapted as agar-overlay methods (Rasoanaivo and Ratsimamanga-Urverg, 1993). However, the differential diffusion of the bioactive compounds from the TLC plate to the agar layer make the method unsuitable for certain class of compounds, especially the water-insoluble types like terpenoids and non-polar compounds (Eloff, 1998). The direct bioautography method allows the localization of a number of components with significant individual inhibitory activities against the tested organisms. The characteristic features of this method are its quickness, efficiency, simplicity, high sample throughput, small test sample size and no sophisticated equipment required. The method is adaptable and applicable to all extracts that can be separated on TLC, against any organism capable of growing directly on TLC plate surfaces.

4.6.2. Minimum inhibitory concentration (MIC)

In this investigation, in vitro antimicrobial efficacy of the crude 70% acetone leaf extracts and fractions derived from 27 plants (13 genera across 9 families) used in South African traditional medicine for treating diarrhoea and related ailments was quantitatively assessed on the basis of minimum inhibitory concentration (MIC). All the plants evaluated exhibited varying degree of inhibitory effect against the standard strain of human and animal pathogenic bacteria (Gram-positive as well as Gram-negative) and clinical isolate of pathogenic fungi. There have been no specific cut-off values as a reference or standard for categorizing antimicrobial activity of plant extracts and fractions. In this study, crude extracts and fractions with an MIC value less than 500 µg/ml were considered to have good activity and MIC value less than 100 µg/ml were considered to have significant

antimicrobial activity of pharmacological interest according to the criterion by Rios and Recio (2005). A lower MIC values indicated high effectiveness of the compound as antimicrobial agent as little quantity which may be below toxicity level of the extracts can be applied without being harmful to the host.

Crude extracts of 4 out 27 had an MIC less than a 100 µg/ml (*Bauhinia bowkeri*, *Bauhinia galpinii*, *and Combretum padoides*) against *E. faecalis* and *Bauhinia petersiana* against *P. aeruginosa* (78 µg/ml). However, the antimicrobial activities were potentiated in the fractions with the non-polar fractions of hexane (MIC ranges from 19 to 312 µg/ml) and dichloromethane (MIC ranges from 19 to 625 µg/ml) enriched with terpenoids exhibiting more broad-based potency compared with the polar fractions of ethyl acetate (MIC ranges from 39 to 1250 µg/ml) and butanol (MIC ranges from 39 to <2500 µg/ml). The MIC value less than 100 µg/ml obtained for some fractions were significant although much higher than that of the control antibiotic (gentamicin with an MIC ranged from 0.18 to 1.56 µg/ml against bacteria and amphotericin B with an MIC ranged from 0.78 to 6.25 µg/ml against fungi).

The water fractions have relatively low antimicrobial activities (MIC ranges from 312 to <2500 µg/ml) except for the *C. vendae* with an MIC value 39 µg/ml against *E. faecalis* and *S. aureus*. In traditional medicine plant preparation, water is used as the major extractant. The poor antimicrobial activity of water fractions of most of the plants indicated that decoction or infusion may be less effective in infectious diseases. These observations are consistent with most of the findings in other studies.

From the phytochemical evaluation, the crude extracts contain high level of polyphenolic compounds. The activity of the extracts and polar fractions (ethyl acetate, butanol, and residual), though not exclusive to polyphenolic compounds only would be expected to correlate to the respective constituents, the structural configuration, functional groups and possible synergistic effects among the constituents. Members of this class of compounds are known to have either bacteriostatic or bactericidal properties against most microorganisms depending on the structure and concentration used. The mechanism of their antimicrobial activity may be related to their fundamental properties of having the ability to form complex with protein and polysaccharides, thus the capacity to inactivate microbial adhesions, enzymes, and cell envelope transport protein. The presence and position of hydroxyl group in the phenolic structure determine and influence the antimicrobial activities of this class of compounds (Taguri *et al*, 2004). Phenolic compounds including tannins and flavonoids were found to have high antimicrobial activity (Majhenic *et al*, 2007; Vaquero *et al*, 2007). Some mechanisms of antimicrobial activity of phenolic compounds includes their ability to denature microbial proteins as surface-active agents (Sousa *et al*, 2006), ability to react with cellular membrane component which impairs both function and integrity of cells (Raccach, 1984), and the reducing property of phenolics can influence the redox potential (E_h) of microbial growth causing growth inhibition (Jay, 1996).

However, two methods are widely used in quantitative evaluation antimicrobial activities of plants extracts: Agar disc diffusion method (NCCLS, 2002) and serial dilution method (Eloff, 1998). Both methods depend on the effective solubility of the extracts in the test medium in order to obtain a maximum efficacy against the organisms. However, some phenolic compounds form complex with proteins and other macromolecules present in the test medium, therefore, get precipitated. While some extract components especially the non-polar are not readily soluble in test medium which is more than 90% water in most cases. These factors may at times cause reduction in the effectiveness of the plant extracts to inhibit microbial growth. The antimicrobial profiles indicated that the extracts and fractions there from were active against Gram-positive and Gram negative bacteria, yeast and mould fungi. The susceptibility of both bacteria and fungi to the extracts may be indicative of the presence of broad-based bioactive compounds or general metabolic toxins.

Pathogenic enteric microorganisms present in contaminated food and water produces enterotoxins or irritants that cause intestinal disorder such as diarrhoea. *In vitro* antimicrobial assays against standard strains of the intestinal pathogens using the polyphenolic-rich crude extracts and fractions have demonstrated various degree of microbial growth inhibition. The plant extracts and fractions investigated have moderate to good activities against diarrhoeal standard strains such as *E. coli*, *S. aureus* and *C. albicans* and *P. aeruginosa*, thus validating their use in traditional medicine for treatment of diarrhoea symptoms. The mechanisms involved in diarrhoea symptoms are multifaceted and interwoven. It is, therefore, possible that extracts and fractions with moderate antimicrobial activities could still have good antidiarrhoeal effects by elaborating other biological activities such as antioxidant, anti-inflammatory, antisecretory, binding of toxins, and antimotility effects on the gastrointestinal tract.

4.7. Conclusion

In infectious diarrhoea many bacteria, protozoa, virus and parasites have been implicated as causative agents. These agents include *Vibrio cholera*, *Escherichia coli*, *Shigella dysenteriae*, *Bacillus cereus*, *Stapylococcus aureus*, *Clostridium difficile*, *Entamoeba histolytica, Salmonella typhi* and *Giardia lamblia*. Some viruses such as Rotavirus and adenovirus have also been implicated as causative agent of diarrheal diseases. The infectious mechanisms of the pathogenic strains of the enteric microbes include microbial adhesion and attachment, localized effacement of the epithelial mucosa lining, production and elaboration of secretory enterotoxins, production of cell-destroying cytotoxins, and direct epithelial cell invasion.

In this study, the emphases were on *E, coli*, *S. aureus*, *E. faecalis*, and *C. albicans* as diarrhoeal pathogens. Seven virulence groups of diarrheagenic *E. coli,* namely enteropathogenic *E. coli* (EPEC), enterohaemorrhagic *E. coli* (EHEC), enteroinvasive *E. coli* (EIEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAggEC), diarrhoea-associated haemolytic *E. coli* (DHEC) and cytolethal distending toxin (CDT)-producing *E.*

coli have been classified (Clarke, 2001). On a global scale EPEC, EHEC, ETEC and EIEC are the most important diarrhoeal agents accounting for 4-8%, 0-1%, 12-20% and 0-2% respectively in terms of total episodes (Bhan, 2000). The virulence mechanisms of ETEC, EHEC, *S. aureus*, *E. faecalis* and some strains of *V. cholerae* include production of endotoxin, cytotoxins and reactive species. The use of antimicrobial therapy with microbicidal or microbiostatic mechanisms may not be effective in the diarrhoea cases involving these organisms because the toxins if already present in contaminated food or water does not need the pathogens to exert activity. Therefore, non-antimicrobial therapy may be required in such cases but antitoxins which can antagonize toxin and receptor interactions. More work is needed in evaluating the antitoxin and antiadhesion of medicinal plant extracts as other forms anti-infectious mechanisms.

Many of the plant extracts and fractions used have good activities especially the non-polar fractions of hexane and dichloromethane against the pathogens tested, and this may explain the traditional use of these medicinal plants.

Considering importance of oxidative burst such as ROS/RNS in the immune mechanisms and possible consequences of cellular damages, if the resultant oxidative stress is not resolved by the endogenous antioxidant system, the antioxidant potentials of the plants will be evaluated in the next chapter.