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 intensivity, 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°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.

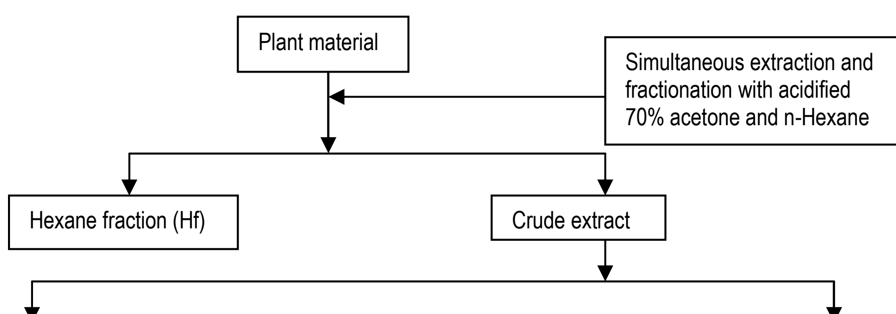
Table 3.1: Medicinal plants selected for antidiarrhoeal investigation in this study

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.



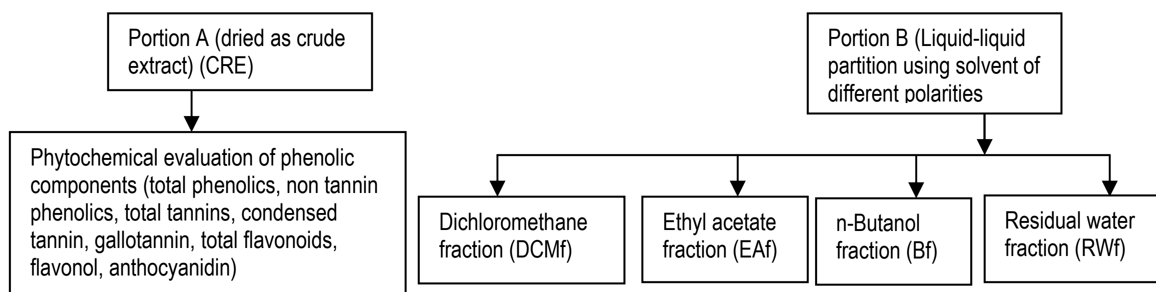


Fig. 3.1. Flow chart for the extraction, phytochemical analysis and fractionation of plant material

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 constituents of the extracts were determined using Folin-Ciocalteau method as described by Makkar 2003, with some modifications. The crude extracts at concentration of 1:1 (mg/ml) plant material: extracting solvent (50 µl) was dispensed into a test tube and made up to 500 µl with distilled water. Folin-Ciocalteau reagent (250 µl) diluted with distilled water (1:1) and 1250 µl of 20% sodium carbonate solution were

added to the extract. The mixture was vortexed and absorbance recorded at 725 nm after 40min incubation at room temperature. The amount of polyphenols (expressed as mg Gallic /g dry weight) was calculated from a prepared standard curve for gallic acid (0.0019-0.25 mg/ml gallic acid). The standard curve equation is y = 4.9022x + c, where y is absorbance, x is mg Gallic acid, c=0, R^2=0.9804)

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 3.7.1 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. To this 3 ml of butanol/HCl (95/5%) 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 50min. The absorbance was recorded at 550 nm after the tubes were allowed to cool 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 vanillin/HCl assay as described by Heimler *et al*, 2006. To 0.5 ml of the extract measured into a test tube, 3 ml of vanillin reagent containing 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 against methanol as blank. The amount of condensed tannin in the extracts was expressed as catechin equivalent (CE)/g dry plant material. The standard curve ranged from 0.0019 to 0.25 mg/ml (Absorbance= 0.1791 mg catechin + 0.0504, R^2=0.944).

3.6.5. Determination of hydrolysable tannin (gallotannin)

The gallotannin content of the extracts was determined using 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. A standard curve was prepared

using gallic acid under the same conditions as the extracts and results were expresses as gallic acid equivalent (GAE)/g dry plant material (Absorbance= 0.8264mg catechin + 0.0392, R^2=0.9155).

3.6.6. Determination of total flavonoids and flavonol

The total flavonoids content of the extracts was determined by aluminium chloride method as described by Abdel-Hamed *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 after 40 mins. Blank samples were prepared with the extract without $AlCl_3$. Standard curve was prepared using quercetin (3.9-500 µg/ml) in methanol under the same condition. The amount of flavonoids was expressed as mg quercetin equivalent/g of dry plant material (Absorbance= 4.9747 mg quercetin, R^2=0.9846).

The flavonol content of the extracts was determined by aluminium chloride method as described by Abdel-Hamed *et al* (2009) with some modification. One ml of the extract was mixed with 1 ml of 20 mg/ml of $AlCl_3$ and 3 ml of 50 mg/ml of CH_3COONa. Standard curve was prepared using quercetin (0.0019 - 0.0312 mg/ml) in methanol under the same condition. Absorbance was read at 440 nm after 2.5 hr. The amount of flavonol was expressed as mg quercetin equivalent/g of dry plant material (Absorbance= 34.046mg quercetin, R^2=0.9853).

3.6.7. Determination of anthocyannin

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 spectrophotometer. 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 equation 1 and 2 (Lee *et al*., 2005).

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

Equation 2 Anthocyanin (cyanidin-3-glucose equivalent mg/L) = $A \times MW \times DF \times 10^3 / \ \times l$

Where MW (molecular weight) = 449.2 g/mol for cyanidin-3-glucose (cyn-3-glu); DF = dilution factor; l = pathlength in cm; = 26900 molar extinction coefficient in $L \times mol^{-1} \times cm^{-1}$ 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*).

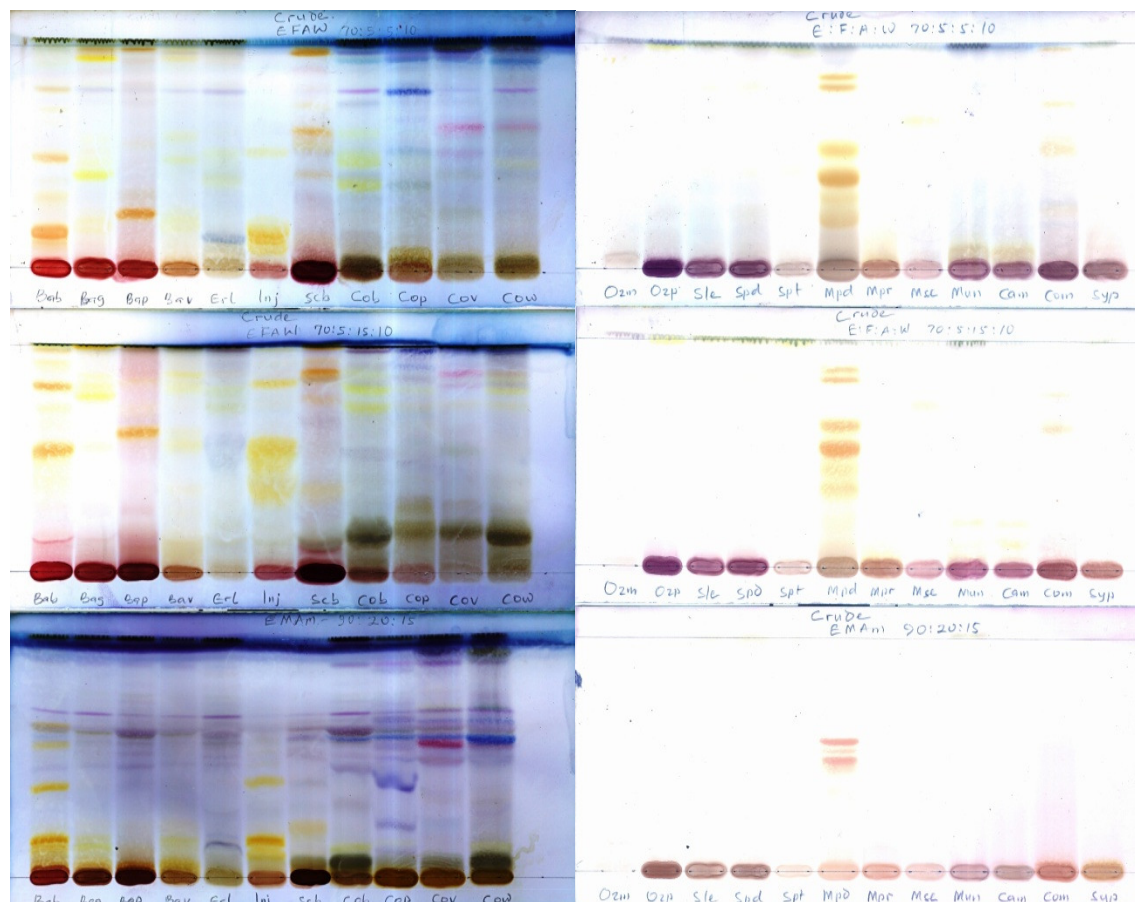


Fig.3.2. Chromatograms of 100 µg of crude extracts of different plant species developed with ethyl actetate: acetic acid: formic acid: water (75:5:5:10) (top), ethyl actetate: acetic acid: formic acid: water (70:5:15:10) (middle) and ethyl acetate: methanol: ammonia (90:20:15) (bottom) and visualized 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 ($AlCl_3/CH_3COOH$).

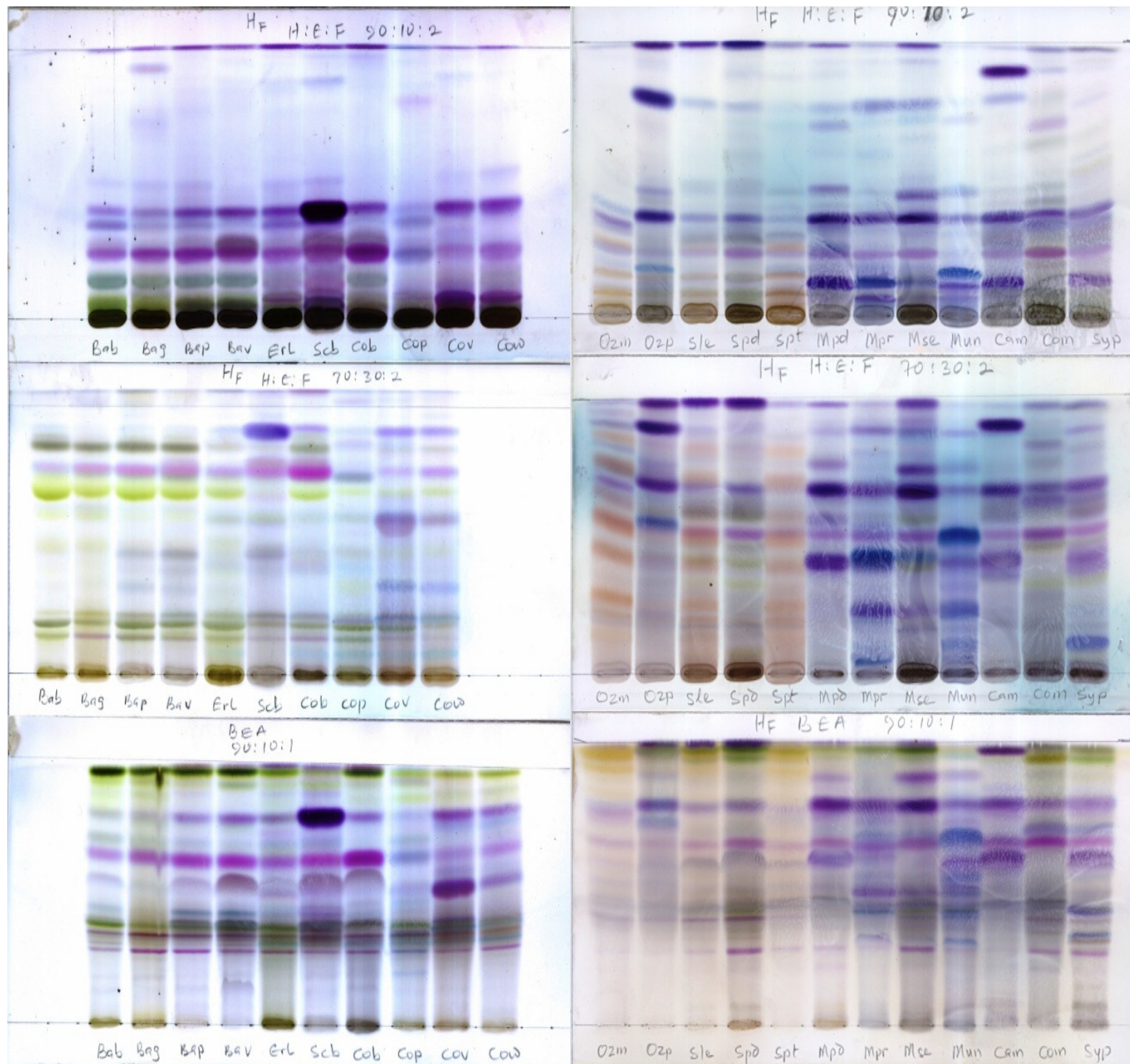


Fig. 3.3: Chromatograms of the hexane fractions of different plant species developed with hexane: ethyl acetae: formic acid (90:10:2) (top), hexane: ethyl acetae: formic acid (70:30:2) (middle), and benzene: ethyl acetate: ammonia (90:10:1) (bottom) and visualized with vanillin sulphuric acid. For identity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

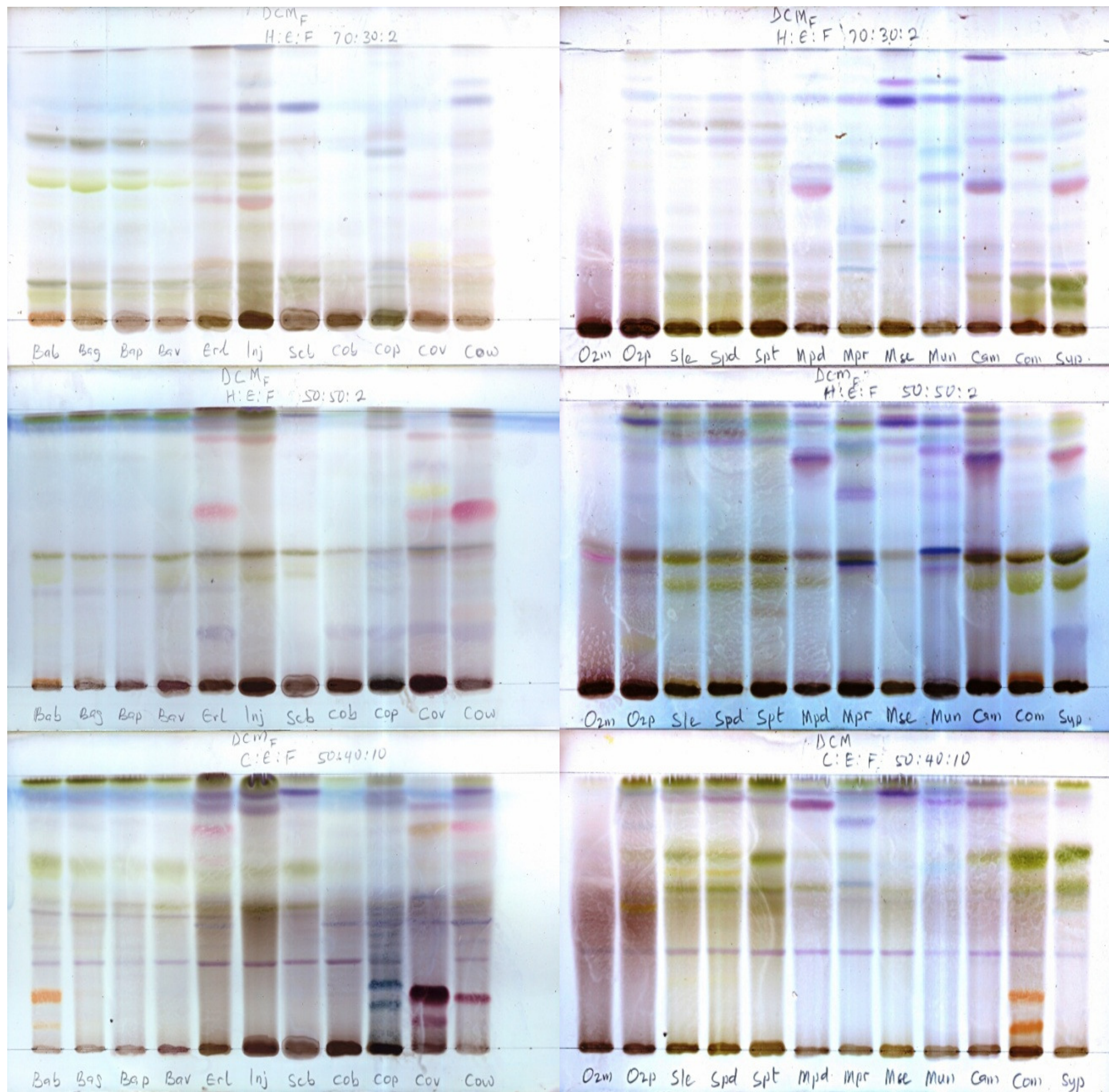


Fig 3.4: Chromatograms of the dichloromethane fractions of different plant species developed with hexane: ethyl acetae: formic acid (70:30:2) top, hexane: ethyl acetae: formic acid (50:50:2) (middle) and chloroform: ethyl acetate: formic acid (50:40:10) bottom and visualized with vanillin sulphuric acid.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

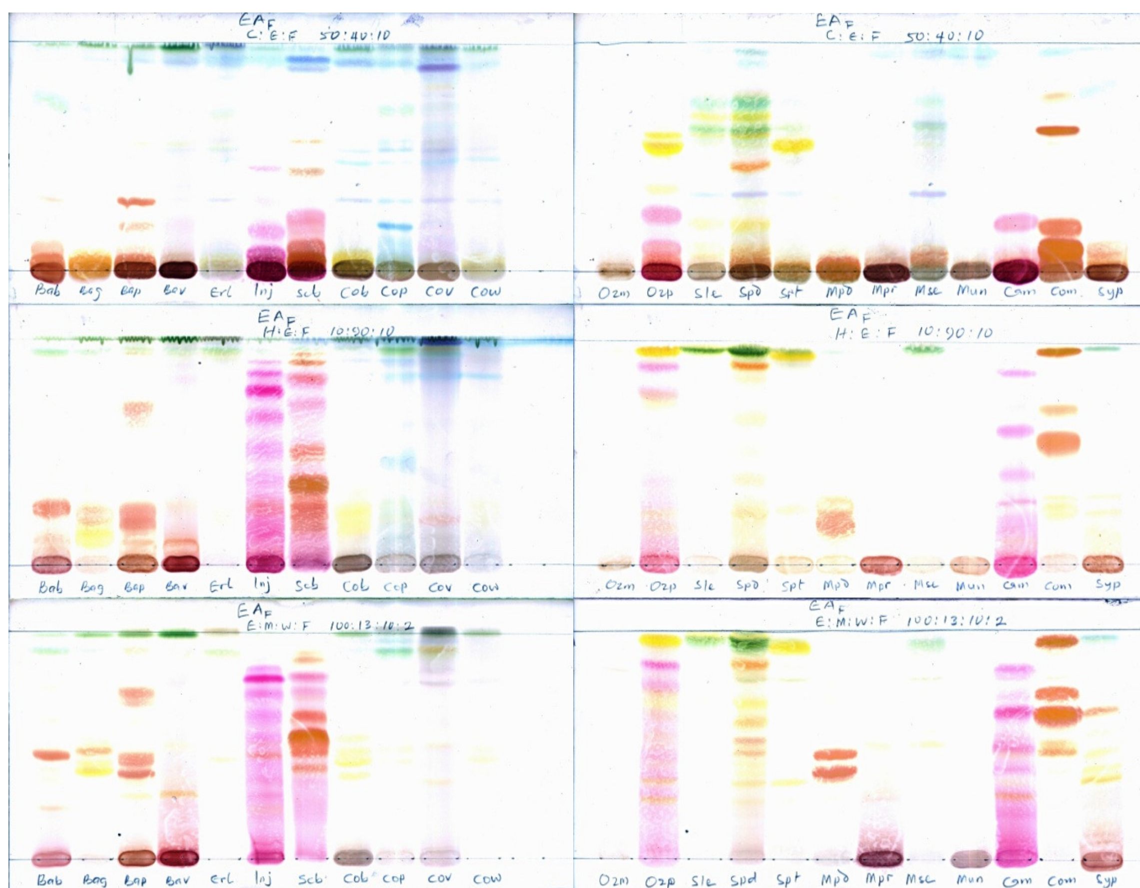


Fig 3.5 Chromatograms of the ethyl acetate fractions of different plant species developed with chloroform:ethyl acetate:formic acid (50:40:10) (top), hexane: ethyl acetate:formic acid (10:90:10) (middle) and ethyl acetate:methanol:water:formic acid (100:13:10:2) (bottom) and visualized with vanillin 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 mgGAE/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 lattisima* 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) $p < 0.05$.

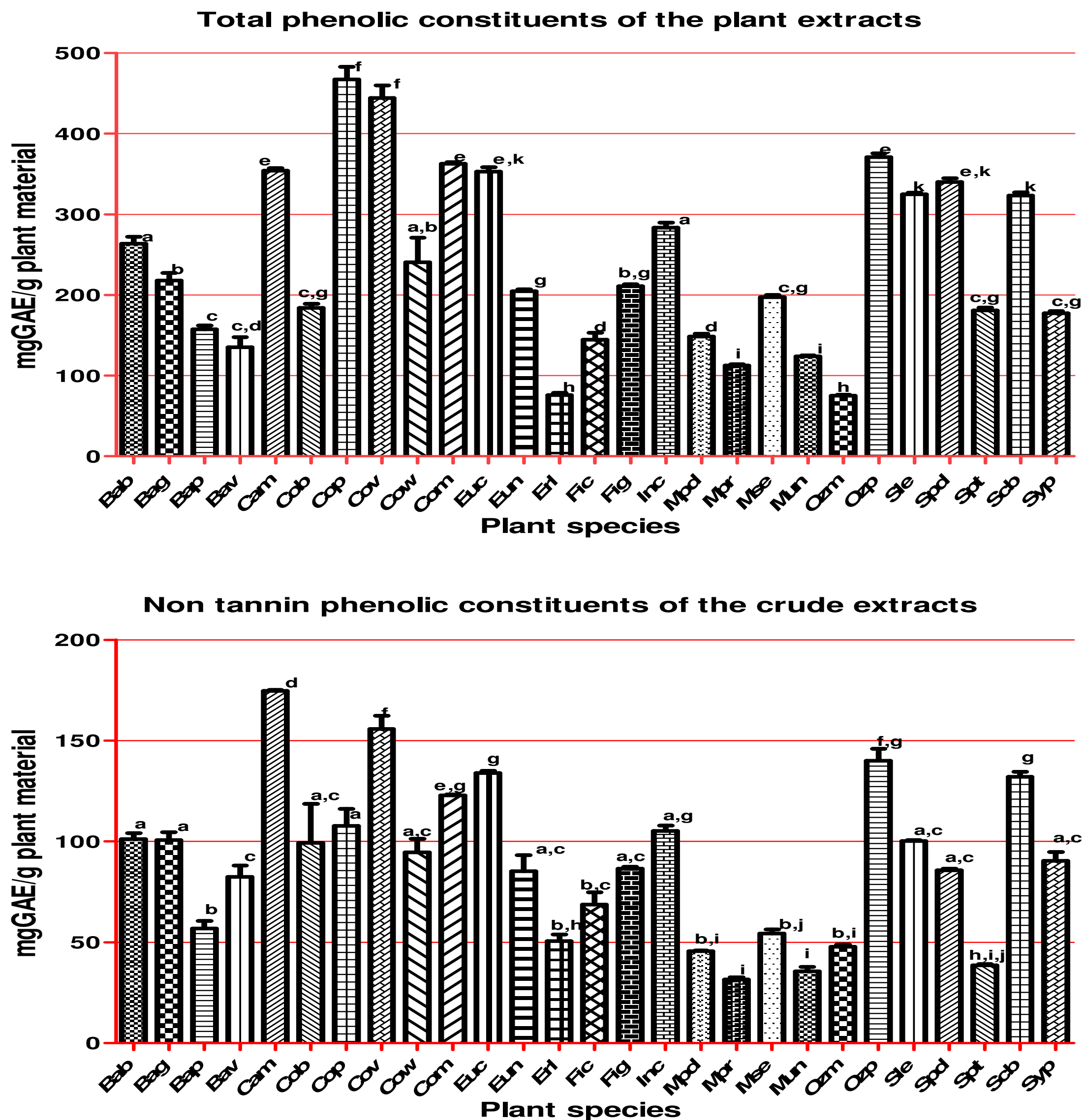


Fig. 3.6. Total phenolic and non tannin constituent of the crude plant extracts

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)

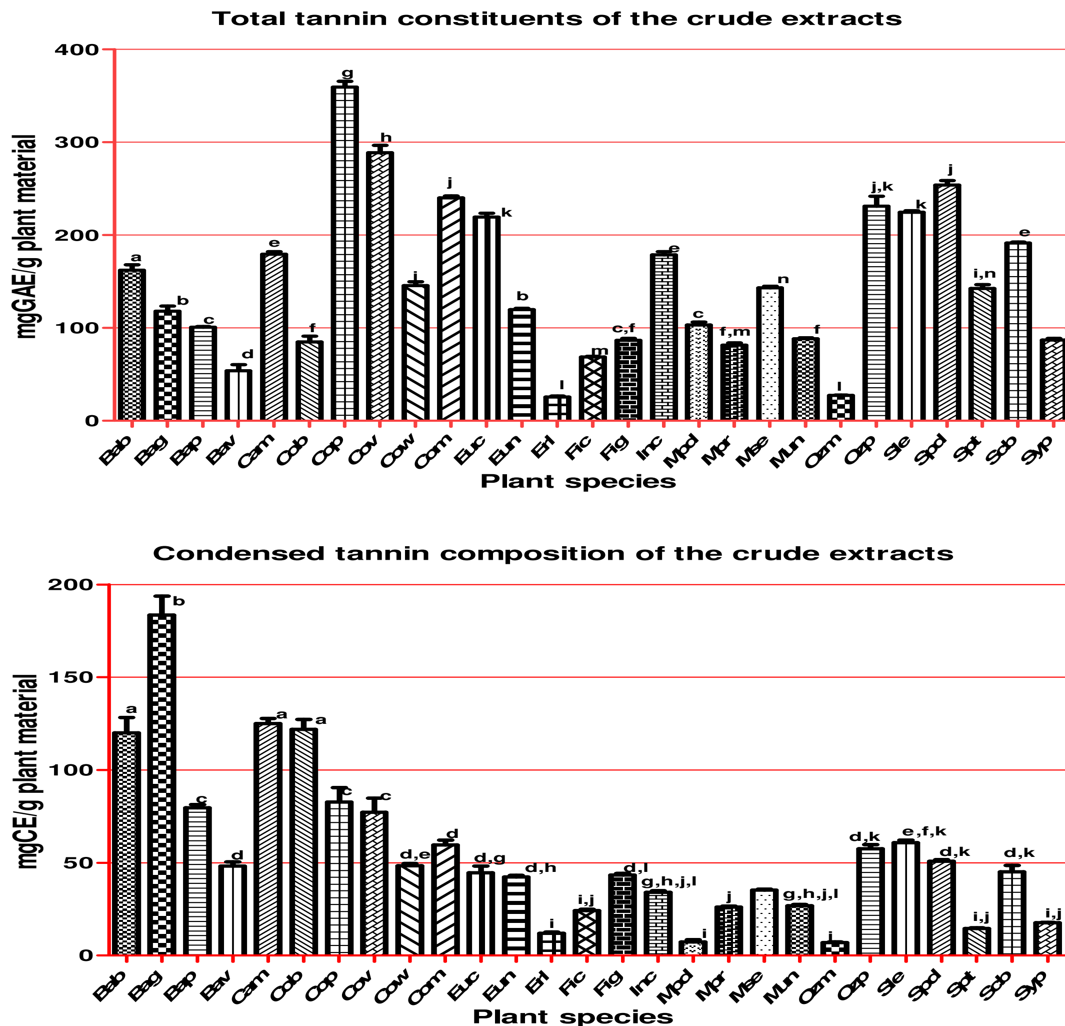


Fig. 3.7. Total tannin and condensed tannin of the crude extracts

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).

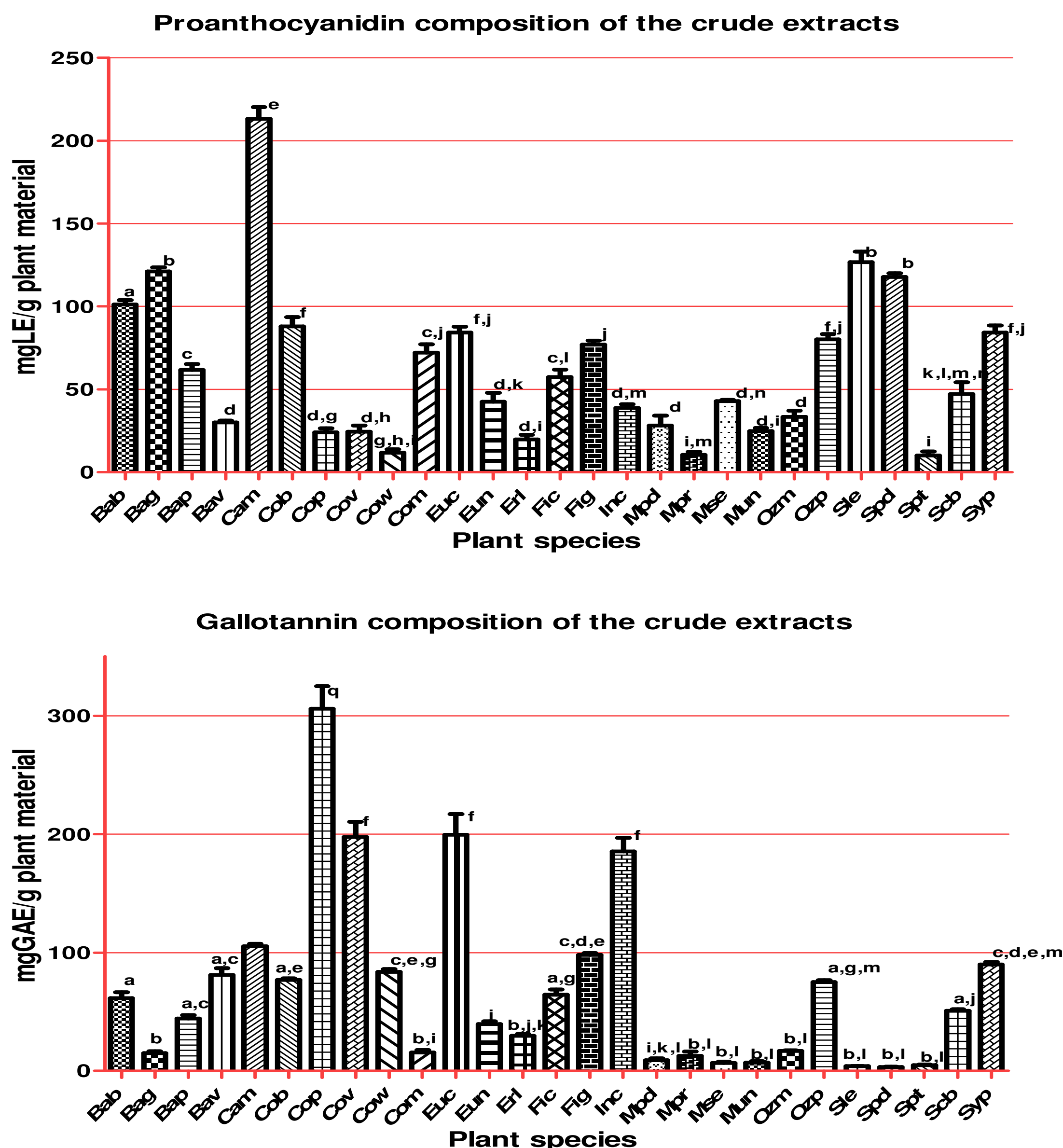


Fig. 3.8. Proanthocyanidin and gallotannin constituent of the crude extracts

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.

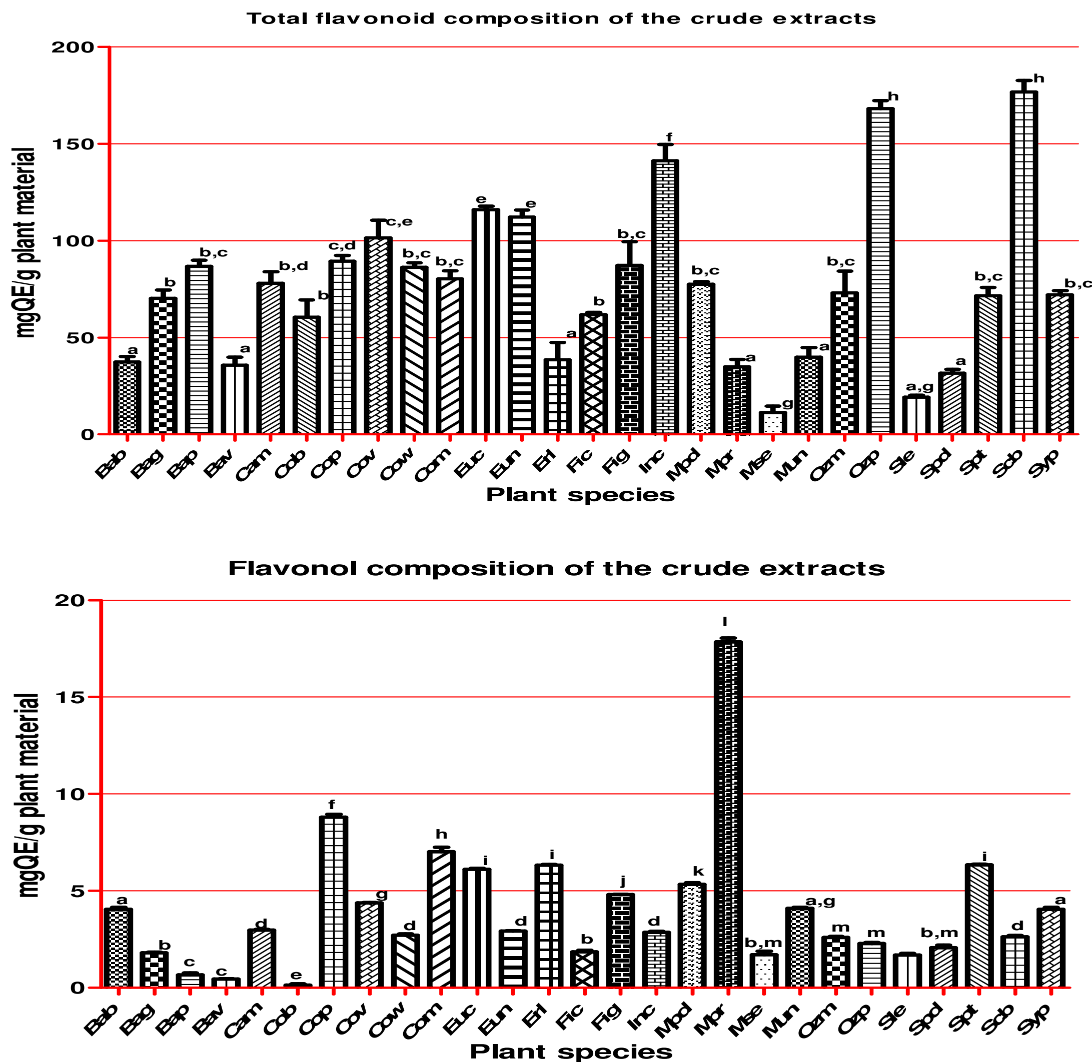


Fig. 3.9. Total flavonoids and the flavonol constituent of the crude extracts

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, $O_2^{\cdot-}$, and $OH^{\cdot}$) and per-oxy-nitrite 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 $O_2^{\cdot-}$ is increased at a rate that overwhelms the capacity of the endogenous SOD enzyme defense to dissipate. Reduction in the $O_2^{\cdot-}$ 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 $O_2^{\cdot-}$ 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.