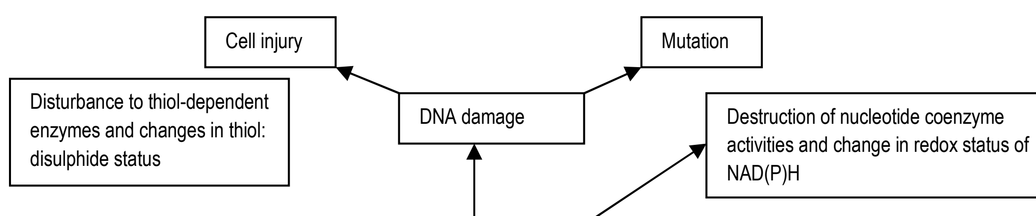
CHAPTER FIVE

Free radical scavenging and antioxidant activities of the extracts and fractions as antidiarrhoeal mechanism.

5.1. Introduction

The intestinal mucosa lining is constantly exposed to the oxidants and toxins from the diet, as well as to endogenous free radicals and other highly reactive species commonly referred to as reactive oxygen species (ROS) or reactive nitrogen species (RNS). These reactive species are generated endogenously in many basic biochemical processes of the body from the respiration (Stojiljkovic *et al*., 2009) and some cell-mediated immune functions (activated neutrophils and macrophages) in response to microbial infection (Neish, 2009). The enzymatic sources of ROS/RNS include NAD(P)H oxidase, xanthine oxidase, uncoupled endothelial nitric oxide (NO) synthase (eNOS), arachidonic acid metabolizing enzymes such as cytochrome P-450 enzymes, lipoxygenase and cyclooxygenase, and the mitochondrial respiratory chain (Griendling, 2005; Mueller *et al*., 2005). Exogenous hazards such as exposure to ionizing radiation, smoke and toxins can also generate free radicals (Masoko and Eloff, 2007; Li and Trush, 1994). Free radicals such as trichloromethyl ($CCl^{\cdot}_3$), superoxide ($O_2^{\cdot -}$), hydroxyl ($^{\cdot}OH$), peroxyl ($ROO^{\cdot}$), and nitric oxide ($NO^{\cdot}$) are produced metabolically in living organisms. In addition, some non-radical derivatives of oxygen molecules (hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl)), are also generated in biological systems. The formation of ROS/RNS have been implicated in the pathogenesis of several human and animal diseases such as atherosclerosis, diabetes mellitus, chronic inflammation, neurodegenerative disorders, gastrointestinal disorders and certain types of cancer (Catalá, 2006).The mechanisms involves in diseases initiation by free radical or oxidative species are outline in Fig 5.1. In physiological conditions, the epithelial mucosa cell integrity and homeostasis are protected from deleterious effects of ROS by antioxidant defence system consisting of nonenzymatic antioxidants (glutathione (GSH), vitamins A, C, E, carotenoids) and antioxidant enzymes superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GR) (Krishnaiah *et al*., 2011). However, in pathological conditions excessive oxidation (oxidative stress) in the intestinal tract result in lipid peroxidation of the membrane phospholipids. The peroxidation of membrane phospholipids is basically damaging because the formation of lipid peroxidation products leads to the spread of free radical reactions and cytotoxic aldehydes by-products. The general process of lipid peroxidation consists of three stages: initiation, propagation, and termination (Catalá, 2006). The initiation phase of lipid peroxidation includes hydrogen atom abstraction. Several species can abstract the first hydrogen atom and include the radicals: hydroxyl ($^{\cdot}OH$), alkoxyl ($RO^{\cdot}$), peroxyl ($ROO^{\cdot}$), and possibly $HO_2^{\cdot}$ but not H_2O_2 or $O_2^{\cdot -}$ (Gutteridge, 1988).



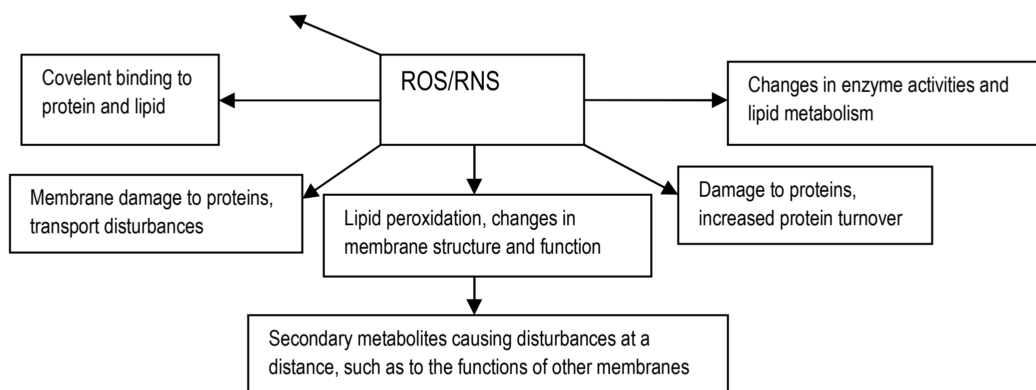


Fig. 5.1. Deleterious reactions from the production of reactive free radicals in biological system (amended from Slater *et al*., 1987)

The initial reaction of ${}^{\bullet}OH$ with polyunsaturated fatty acids produces a lipid radical ($L^{\bullet}$), which in turn reacts with molecular oxygen to form a lipid peroxyl radical ($LOO^{\bullet}$). The $LOO^{\bullet}$ can abstract hydrogen from an adjacent fatty acid to produce a lipid hydroperoxide (LOOH) and a second lipid radical (Catalá, 2006). The LOOH formed can suffer reductive cleavage in the presence reduced metals, such as Fe^{2+}, producing lipid alkoxyl radical ($LO^{\bullet}$). Both alkoxyl and peroxyl radicals stimulate the chain reaction of lipid peroxidation by abstracting additional hydrogen atoms (Buettner, 1993) (See Fig. 2.6 for detailed reaction mechanisms). Peroxidation of lipids can disturb the assembly of the membrane, causing changes in fluidity and permeability, alterations of ion transport and inhibition of metabolic processes (Nigam and Schewe, 2000). Injure to mitochondria induced by lipid peroxidation causes further ROS generation (Green and Reed, 1998). In addition, LOOH break down, frequently in the presence of reduced metals or ascorbate, to reactive aldehyde products, including malondialdehyde (MDA), 4-hydroxy-2-nonenal (HNE), 4-hydroxy-2-hexenal (4-HHE) and acrolein (Esterbauer *et al*., 1991; Parola *et al*., 1999; Uchida, 1999; Kehrer and Biswal, 2000; Lee *et al*., 2001).

Lipid peroxidation and the metabolites are the main oxidative biochemical processes contributing to the disruption of detoxifying pathways in intestine and to dysfunction of enterocytes, which may cause various disorders of digestive tract including diarrhoea. GSH redox cycle plays the main role in lipid peroxidation scavenging in the intestine. Enzyme GPx reduces LOOH using GSH as a reducing factor, while GR regenerates GSH from its oxidized form glutathione disulfide (GSSG), with simultaneous oxidation of nicotinamide adenine dinucleotide phosphate (NADPH). GSH acts not only as an enzyme cofactor, but can react directly with free radicals and is involved in recycling other cellular antioxidants. Excessive generation of ROS/RNS and depleted endogenous antioxidant defences have been implicated in the pathogenesis and perpetuation of intestinal damage which can clinically manifest as diarrhoea. Under disease conditions, more ROS/RNS is generated by the body enhancing the oxidative stress. ROS are also effective in activating redox-responsive pro-inflammatory transcription factors, nuclear factor (NF)-β and activator protein (AP)-1 (Rahman and Adcock, 2006).

Supplementary therapy with antioxidant compounds provides an additional relief against deleterious effect of ROS/RNS.

5.1.1. Superoxide ion

Superoxide radical anion ($O_2^{\bullet-}$) generated from an electron leakage in the mitochondrial respiration chain and the conversion of xanthine dehydrogenase to xanthine oxidase (Wernes and Lucchesi, 1990) as a result of electron donation to oxygen molecule is regarded as the primary ROS in biological system. Although $O_2^{\bullet-}$ is not very active but the radical interact with other molecules to produce highly potent secondary ROS either directly or indirectly through enzyme and/or metal catalyzed mechanisms (Valko *et al*., 2005). 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.

5.1.2. Hydrogen peroxide

The generation of hydrogen peroxide (H_2O_2) by activated phagocytes plays an important part in the killing of several bacterial and fungal strains (Sanchez-Moreno, 2002). Additionally, H_2O_2 is generated *in vivo* under physiological conditions by peroxisomes and several oxidative enzymes including glucose oxidase, d-amino acid oxidase, and dismutation of superoxide radical, catalysed by superoxide dismutase. There is increasing evidence that H_2O_2, either directly or indirectly via its reduction product (hydroxyl ion (OH^-)), acts as a messenger molecule in the synthesis and activation of inflammatory mediators (Auroma *et al*., 1989).

5.1.3. Hydroxyl radical

Hydroxyl radical ($^{\bullet}OH$) is the neutral form of hydroxyl ion and the most reactive free radical in biological systems generated from free metal ions (copper or iron) catalyzed breakdown of H_2O_2 (Fenton reaction) or superoxide ion reaction with H_2O_2 (Haber-Weiss reaction, Fig 2.6). Hydroxyl radicals have short half of 10^{-9} s with the highest 1-electron reduction potential of 2310 mV, and is primarily responsible for the cytotoxic effect in aerobic organism. The radical reacts with every cell components in living organisms at the second-order rate constants of 10^9–10^{10} mol/s (Siddhuraju and Becker, 2007) such as lipid, polypeptides, proteins, and DNA, especially thiamine and guanosine. Unlike $O_2^{\bullet-}$ and H_2O_2, which can be enzymatically eradicated by the activity of superoxide dismutases ($2O_2^{\bullet-} + 2H^+ \rightarrow H_2O_2 + O_2$) and catalases/peroxidises ($2H_2O_2 \rightarrow 2H_2O + O_2$), respectively, there exists no known enzyme that catalyzes the cellular detoxification of $^{\bullet}OH$ (Dwyer *et al*., 2009).

5.1.4. Peroxyl radical

Peroxyl radicals are important reactive species in living systems formed by a direct reaction of oxygen with alkyl radicals or the protonation of the superoxide ions. Peroxyl radicals are potent oxidants with standard reduction potential of more than 1000 mV (Decker, 1998). The radicals abstract hydrogen from other molecules with lower standard reduction potential to perpetuate chain reaction such as propagation stage of lipid peroxidation. Cell

membranes including intestinal epithelial mucosa are phospholipid bilayers with extrinsic proteins and are the primary target of lipid peroxidation (Girotti, 1998) causing cell dysfunction and tissue injury. Lipid peroxidation cytotoxic by-products such as malonaldehyde can react with free amino group of proteins, phospholipid, and nucleic acids leading to structural modification, which induce dysfunction of immune systems.

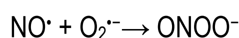
5.1.5. Hypochlorous acid

Hypochlorous acid (HOCl) is also a strong oxidant generated *in vivo* by neutrophil myeloperoxidase (MPO) catalyzed oxidation of chloride ions and H_2O_2. The cytotoxicity of this reaction contributes to the phagocytosis of infectious microorganisms in the host defence system. However, HOCl generated by MPO also inactivate some enzymes such as α-antiproteinase contributing to proteolytic damage of healthy human tissues in inflammatory disease (Halliwell and Gutteridge, 1990; Hippeli and Elstner, 1999). The oxidant has also been implicated as a secretagogue.

5.1.6. Nitric oxide

Biological tissues generate nitric oxide ($NO^{\bullet}$) by specific nitric oxide synthases (eNOS, iNOS) metabolization of arginine to citrulline via a five electron oxidative mechanisms. In normal physiological processes, nitric oxide ($NO^{\bullet}$) acts as an important oxidative biological signalling molecule in neurotransmission, blood pressure regulation, defence mechanisms, smooth muscle relaxation and immune regulation (Bergendi *et al*, 1999). Nitric oxide ($NO^{\bullet}$) has greater stability in an environment with a lower oxygen concentration compared to the hydroxyl radical with half life >15 s.

Cells of the immune system produce both the superoxide anion and nitric oxide in the oxidative burst inducing inflammatory processes. Under these conditions, nitric oxide and the superoxide anion may react together to produce significant amounts highly reactive oxidative molecule (peroxynitrite anion ($ONOO^-$)). This potent oxidising agent that can cause DNA fragmentation and initiate lipid peroxidation (Carr *et al*., 2000):



$$NO^{\bullet} + O_2^{\bullet -} \rightarrow ONOO^-$$

The $NO^{\bullet}$ toxicity is predominantly linked to its ability to combine with superoxide anions with the rate constants known for reactions of $NO^{\bullet}$, $7.0\times10^9 M^{-1} s^{-1}$.

5.2. Antioxidant assays

Several standardized methods have been proposed to analyze the antioxidant potential of a substrate including plant extract and isolated compounds from it. Criteria for the standard methods include (i) measurement of the chemical process actually occurring in potential applications; (ii) utilization of biological relevant molecules; (iii) technically simple; (iv) with a defined endpoint and chemical mechanism; (v) readily available instrumentation; (vi) good repeatability and reproducibility; (vii) adaptable for assay of both hydrophilic and lipophilic antioxidants; (viii) and adaptable to high-throughput analysis (Prior, *et al*., 2005). The assays are based on scavenging capacity against specific biological ROS/RNS and/or against stable, non-biological radicals and evaluation of

total reduction capacity such as 1,1-diphenyl-2-picrylhydrazine (DPPH) radical scavenging assay (Brand-Williams et al., 1995); 2,2'-azinobis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) radical scavenging method (Re *et al*., 1999); β-carotene linoleic acid bleaching assay (Siddhuraju and Becker, 2003); inhibition of linoleic acid peroxidation (Osawa and Namiki, 1981); ferric reducing antioxidant power (FRAP) (Benzie and Szeto, 1999); total radical trapping antioxidant potential (TRAP) assay (Leontowicz *et al*., 2002); hydroxyl radical scavenging activity (Jodynis-Liebert *et al*., 1999); hydrogen peroxide scavenging activity (Ruch et al., 1989); nitro blue tetrazolium (NBT) reduction assay or superoxide anion scavenging activity (Kirby and Schmidt, 1997) and oxygen radical absorbance capacity (ORAC) assay (Silva et al., 2007). Each method has its own merit and demerit in evaluating antioxidant capacity of plant extracts and their components. Based on the criteria enumerated above, the most common and reliable methods are the ABTS and DPPH methods.

5.2.1. Antioxidant bioautography

For the qualitative detection of free radical scavengers and the number of antioxidant compounds present, DPPH or β-carotene is usually the spraying reagents (Martson, 2011) of TLC chromatograms. DPPH is a purple-coloured free radical that turns yellow on reduction by an antioxidative component of an extract. Yellow spots on TLC plates spayed with DPPH solution against the purple background indicate the presence of an active compound(s). In the β-carotene assay, the TLC plate is sprayed with a solution of β- carotene, dried and exposed to 254 nm UV light to bleach the β- carotene. Areas where antioxidants inhibit degradation of β-carotene appear as orange zones on a pale background.

5.2.2. The chemistry of some common antioxidant assays

5.2.2.1. Hydroxyl radical

Hydroxyl radical is the most reactive species and source of many other secondary free radicals in biological systems; thus, it is important to evaluate hydroxyl radical scavenging capability. Hydroxyl radical ($HO^{\bullet}$) scavenging is usually evaluated using the “deoxyribose assay”: a mixture of ferric chloride ($FeCl_3$) and ethylenediamine tetraacetic acid (EDTA) in the presence of ascorbate reacts to form iron(II)-EDTA plus oxidized ascorbate, H_2O_2 then reacts with iron(II)-EDTA to generate iron(III)-EDTA plus $HO^{\bullet}$ from the Fenton reaction ($Fe_2+H_2O_2 \rightarrow Fe_3+ HO^{\bullet} + HO^{-}$). The radicals not scavenged by other components of the reaction mixture attack the sugar deoxyribose, and degrade it into a series of fragments, some or all of which react on heating with thiobarbituric acid at low pH to give a pink chromogen. Thus the scavenging activity towards HO^{-} of a substance added to the reaction mixture is measured on the basis of the inhibition of the degradation of deoxyribose.

Another spectrophotometric method developed for assessment of hydroxyl radical scavenging capacity of antioxidants includes Fenton reaction as the hydroxyl radical generation system and salicylate as a spectrophotometric indicator. Attack by $^{\bullet}OH$ radicals on salicylate produce 2, 3-dihydroxybenzoate, 2, 4-dihydroxybenzoate, and 2, 5-dihydroxybenzoate as major products. The hydroxylated products can be identified and quantified by Beer’s law testing the additivity of absorbances of the hydroxybenzoates. This method is able

to measure the hydroxyl radical scavenging capability of individual antioxidants with a wide dynamic activity range, i.e., 635-637 nm. These spectrophotometric methods may not be the most sensitive, but they are simple, reproducible, and cost effective method valuable in antioxidant studies.

5.2.2.2. Hydrogen peroxide scavenging

Hydrogen peroxide-scavenging activity is measured by using a peroxidase-based assay system. The most common used peroxidase is horseradish, which uses H_2O_2 to oxidize scopoletin into a nonfluorescent product. In the presence of a putative scavenger, the oxidation of scopoletin is inhibited and the H_2O_2 scavenging can be monitored by decay in H_2O_2 concentration spectrophotometrically from absorption at 230 nm using the molar optical density of 81 $M^{-1}cm^{-1}$.

5.2.2.3. Superoxide scavenging capacity

The scavenging activity towards superoxide by antioxidants is measured by the inhibition of generation of superoxide with the hypoxanthine–xanthine oxidase (HX-XO) system. The superoxide generating from HX–XO reduces nitro-blue tetrazolium (NBT) to formazan at pH 7.4 and room temperature which can be followed spectrophotometrically at 560 nm. Any added molecule capable of reacting with superoxide inhibits the production of formazan and the reduction of the absorbance is estimated as superoxide scavenging activity compared to the value obtained with no test added sample.

5.2.2.4. 2, 2-diphenyl-1-picrylhydrazyl radical ($DPPH^{\bullet}$ assay)

The DPPH radical scavenging assay is hydrogen atom transfer processes widely used evaluate the antioxidant activity of reductants (plant extracts, phytochemical or pharmaceutical drugs) (Kaviarasan et al., 2007). Although DPPH assay has no direct biological relevance, the process is related to the inhibition of lipid peroxidation (Rekka and Kourounakis, 1991). The DPPH free radical is reduced to the corresponding hydrazine when it reacts with hydrogen donors (antioxidant) using decolouration mechanisms (purple to yellow), which are monitored by the decrease in absorbance at 515–528 nm. From the methodological point of view the assay conducted in ethanol or methanol solution of DPPH is considered a valid, easy and accurate assay to evaluate radical scavenging activity of antioxidants, since the radical compound is stable and does not have to be generated as in other radical scavenging assays. The results are highly reproducible and comparable to other free radical scavenging methods such as ABTS.

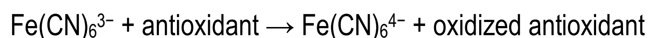
5.2.2.5. 2, 2'-azinobis-(3-ethylbenzothiazoline-6-sulphonate) radical cation ($ABTS^{\bullet+}$) free radical-scavenging method

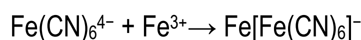
The ABTS radical scavenging method is based on the reduction of blue/green $ABTS^{\bullet+}$ chromophore generated from the reaction between ABTS and potassium persulphate ($K_2S_2O_7$) by an electron-donating antioxidant. The

decolourization of the ABTS$^{\bullet+}$ chromophore is measured spectrophotometrically at 734 nm in both lipophilic and hydrophilic medium.

5.2.2.6. Ferric reducing antioxidant power (FRAP)

The FRAP method is based on the reduction at low pH, of a colourless ferric complex to an intense blue-coloured ferrous complex by an electron donating antioxidant. The reduction of ferric complex is monitored by measuring the increase in absorbance at 750 nm. The chemistry of FRAP assay can be summarized with equation (1) with oxidant accepting an electron from antioxidant to be reduced an intense coloured molecule.





The FRAP assay is a robust and potentially useful test using inexpensive reagents and equipment and a speedy reaction applicable over a wide concentration range.

5.3. Materials and Methods

5.3.1. Qualitative antioxidant assay using TLC-DPPH method

This was done to determine the number of active compound(s) present in the crude extracts and fraction(s) with free radical scavenging capacity against DPPH radical. Chromatograms were developed as described in section 3.6.4 and sprayed with 0.2% methanolic DPPH solution. The presence of yellow spots against a purple background indicated component(s) with antioxidant activities.

5.3.2.1. DPPH$^{\bullet}$ radical-scavenging assay

The antioxidant activities of the samples were measured in term of radical scavenging ability using the stable radical (DPPH$^{\cdot}$) of Brand-Williams *et al*, (1994) with some modifications. Methanol solutions (40 µl) of the samples at various concentrations (19–2000 µg/ml), and positive control (trolox and ascorbic acid) at concentration (19 to 250 µg/ml) were added to 160 µl of DPPH in methanol (25 µg/ml) in a 96 well-microtitre plate. The change in absorbance (516 nm) measured after at 1, 10, 20 and 30 min (A_t) with a microtitre plate reader (Versamax). The sample concentrations were corrected for the dilutions.

5.3.2.2. ABTS$^{\bullet+}$ radical-scavenging assay

The free radical-scavenging activity as a measure of hydrogen donating capacity was determined by using ABTS cation decolourization method of Re *et al.,* 1999 with some modifications. ABTS radical solution (7 µM) was prepared by dissolving 1.32× 10^4 µg of ABTS in 10 ml of 50% methanolic solution and 7.68 ×10^4 µg of potassium persulphate ($K_2S_2O_4$) in 10 ml of distilled water. The two solutions were mixed together and made up to 200 ml with 50% methanolic solution, and kept in the dark at room temperature for 12 h. Prior to running the assay, the ABTS radical solution was diluted with a 50% methanolic solution to an absorbance (A_{o2}) between

0.7- 0.8 at 734 nm. The extracts were serially diluted (40 µl) (19 to 2000 µg/ml) in 96 well-microtitre plate and 160 µl of ABTS radical solution added to each well. The absorbance were taken exactly after 6 min of reaction (A_{t2}) and blank absorbance (A_{b2}) were prepared using the respective extracts without ABTS radical.

5.3.2.3. FRAP assay

The FRAP of the samples was determined by direct reduction of potassium ferric cyanide ($K_3Fe_3(CN)_6$) to potassium ferrocyanide ($K_3Fe_2(CN)_6$) (electron transfer process from the antioxidant). The increase in absorbance from the formation of Pearl's Prussian blue complex following the addition of excess ferric ion was measured as described by Berker *et al*., (2007) with some modification. The reaction medium (200 µl) containing 40 µl of the test samples or positive controls (trolox and ascorbic acid) (concentration range between 19 -2000 µg/ml), 100 µl of 1.0M hydrochloric acid, 20 µl of 1% (w/v) of SDS, 30 µl of 1% (w/v) of potassium ferric cyanide was incubated for 20 min at 50°C, cooled to room temperature and finally 20 µl of 0.1% (w/v) of ferric chloride was added. The absorbance at 750 nm was read and blank absorbance was taken by preparing the reaction medium the same way except the addition of ferric chloride. The reducing capacities were taken as slope obtained from the line of best fit of the absorbance against concentration using the linear regression curve.

5.3.2.4. Hydroxyl radical scavenging assay

The hydroxyl radical scavenging activities of the test samples were measured by the salicylic acid method. The hydroxyl radical scavenging activity of the extracts was determined according to method of Smirnorff and Cumbes, (1996) with some modifications. The hydroxyl radical was generated by using Fenton reaction which contains 50 ml of $FeCl_3$ (8.0 mM), 80 ml of H_2O_2 and 50 ml of distilled water was allowed to stand for 1h. The mixture was filtered to remove the debris. Hydroxyl radical was determined by mixing 120 µl of the hydroxyl radical solution with 66µl of the extracts followed by 14 µl of salicylic acid (20 mM). The mixture was incubated for 30min at 37°C and absorbance taken at 510 nm.

5.3.2.5. Lipid peroxidation inhibition assay

Lipid peroxidation of linoleic acid was determined as described by Kishida *et al*., 1993. Each reaction mixture contained 4.1 ml of 2.5% linoleic acid in ethanol and 10 ml of 0.2M phosphate buffers (pH 7.4), 1.0 ml of 0.0025 mg/ml $FeSO_4$ was added as catalyst. Different concnetartions of the samples (10-500 ug/ml) were added to the reaction mixture in a centrifuge tube. The reaction mixture was incubated at 40°C for 2 h. The reaction was terminated by adding 1.0 ml of 25% trichloroacetic acid and 1.0 ml of 0.67% thiobarbituric acid. The reaction mixture was heated at 95°C for 30 min and cooled for 15 min. The mixture was extracted with butanol and absorbance measured at 500 nm.

5.4. Results

5.4.1. TLC-DPPH analyses (Antioxidant bioautography)

The qualitative antioxidant screening of spraying DPPH on TLC plate indicated the presence of a number of antioxidant compounds in the crude extracts and fractions (hexane, dichloromethane ethyl acetate, and butanol). Antioxidant compounds were visualized as yellow spot against the purple background of DPPH as shown in Fig. 5.2 - 5.5. The numbers of active compounds identifiable on a plate depend on the mobile phase used in the development of the plate. For the crude extracts, solvents 1and 2 separated the antioxidative compounds but solvent 3 revealed close similarity between *Combretum vendae* and *Combretum woodii*, both exhibiting activities at three different spots of same with R_f values of 0.94, 0.53 and 0.48 respectively.

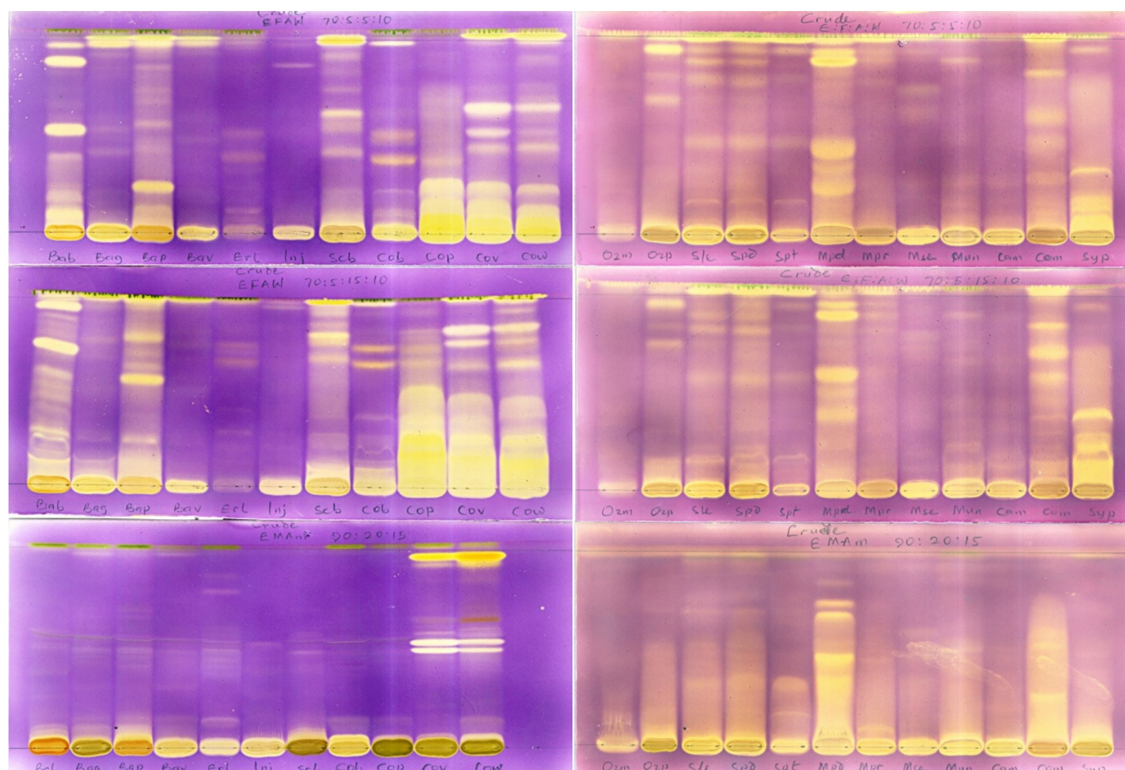


Fig.5.2. TLC-DPPH profiles of the crude extracts of extracts of different plants (left to right **Bab** (*Bauhinia bowkeri*), **Bag** (*Bauhinia galpinii*), **Bap** (*Bauhinia petersiana*), **Bav** (*Bauhinia variegata*), **Erl** (*Erythrina latissima*), **Inc** (*Indigofera cylindrica*), **Scb** (*Schotia 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*) 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)).

For the hexane fraction using eluent 5, the antioxidant compound was present only in *Erythrina latissima* sample with R_f value of 0.26. The dichloromethane fraction of the extract exhibited antioxidant activity at many spots with some being minor while *E. latissima*, *C. vendae*, and *C. woodii* revealed major antioxidant spots at R_f values of 0.6, 0.46 and 0.33 using solvent 5. Spots at R_f values of 0.53 and 0.40 were peculiar to *C. vendae*, and *C. woodii* respectively. Eluent 6 separated more antioxidant components in all the plants tested with *Bauhinia bowkeri*, *E.*

latissima, *C. vendae*, and *C. woodii* exhibiting activity at 0.8, *E. latissima*, *C. vendae*, and *C. woodii* at 0.66, *B. bowkeri*, *B. galpinii*, *B. petersiana*, *B. variegata*, *E. latissima*, *C. vendae*, and *C. woodii* at 0.51, *C. vendae* and *C. woodii* at 0.17 and *C. vendae* at 0.1.

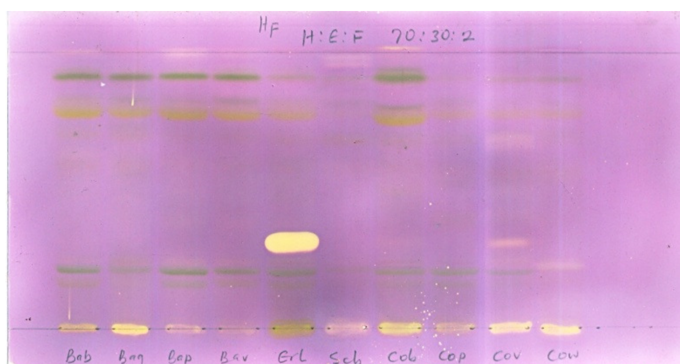


Fig. 5.3. TLC-DPPH profile of the hexane fractions of different plants (**Bab** (*Bauhinia bowkeri*), **Bag** (*Bauhinia galpinii*), **Bap** (*Bauhinia petersiana*), **Bav** (*Bauhinia variegata*), **Erl** (*Erythrina latissima*), **Inc** (*Indigofera cylindrica*), **Scb** (*Schotia brachypetala*), **Cob** (*Combretum bracteosum*), **Cop** (*Combretum padoides*), **Cov** (*Combretum vendae*), and **Cow** (*Combretum woodii*) developed with hexane: ethyl acetae: formic acid (70:30:2))

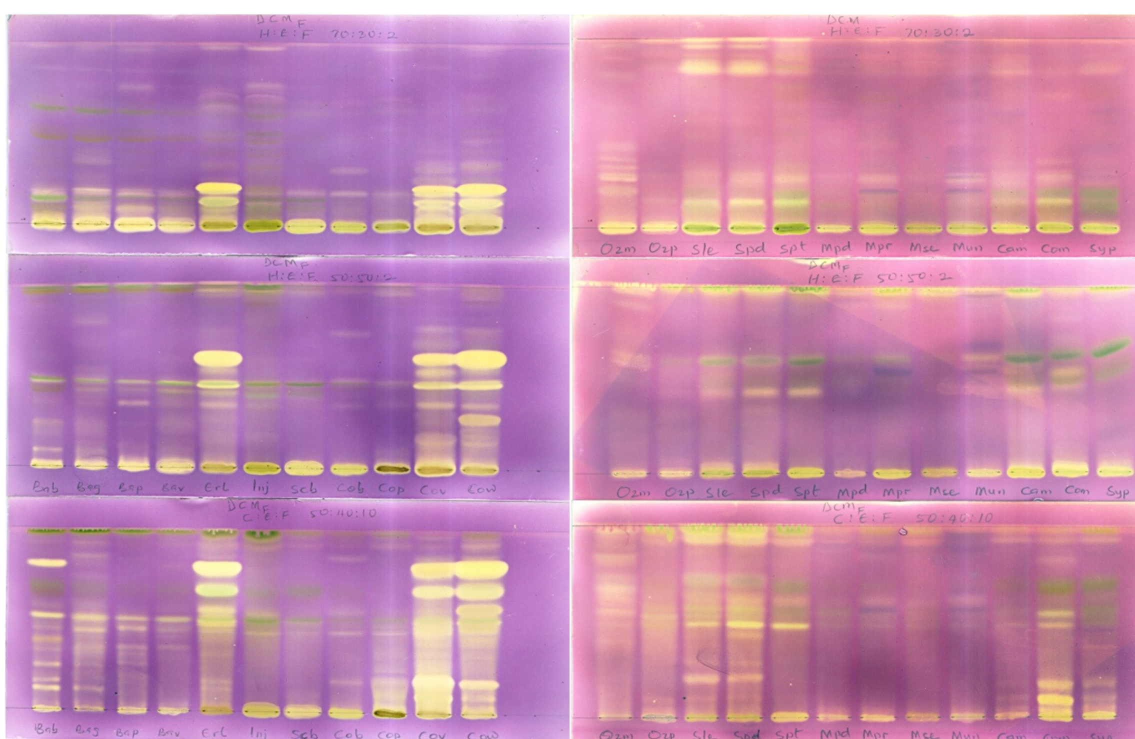


Fig.5.4 TLC-DPPH profiles of the dichloromethane fractions of different plants (left to right **Bab** (*Bauhinia bowkeri*), **Bag** (*Bauhinia galpinii*), **Bap** (*Bauhinia petersiana*), **Bav** (*Bauhinia variegata*), **Erl** (*Erythrina latissima*), **Inc** (*Indigofera cylindrica*), **Scb** (*Schotia 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*) 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.

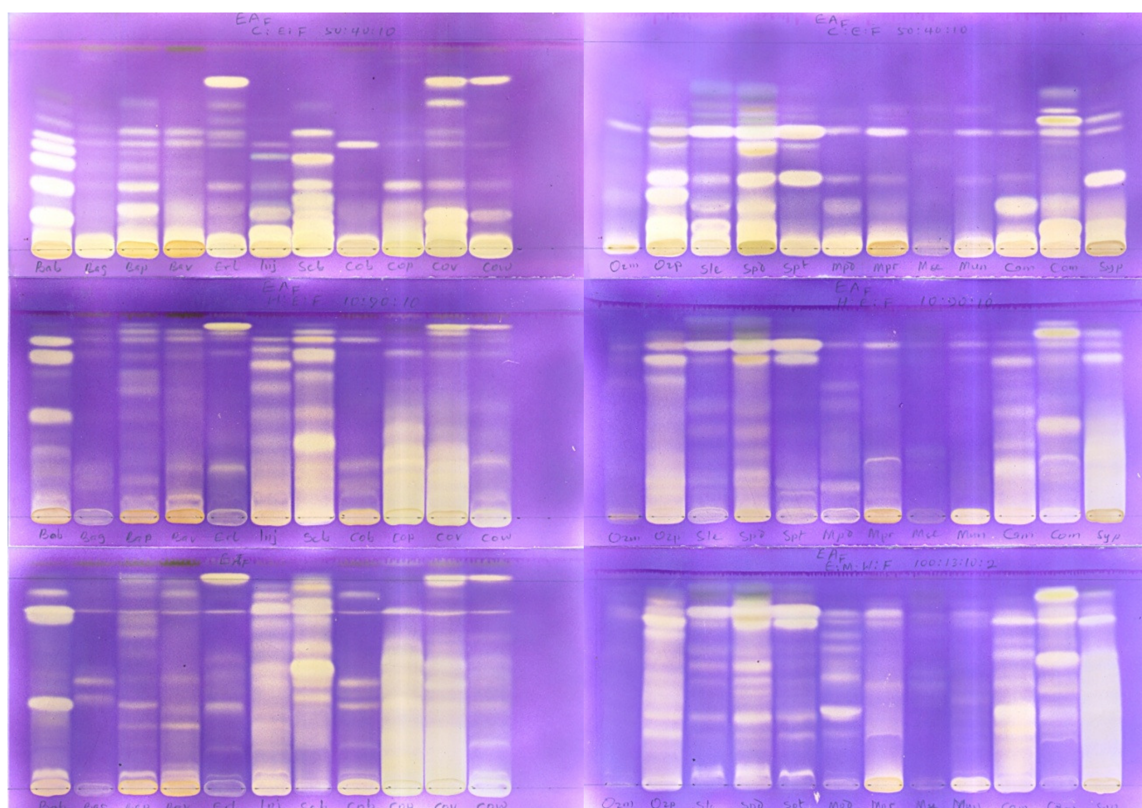


Fig. 5.5. TLC-DPPH profiles of the ethyl acetate fractions of different plants left tor right (left to right **Bab** (*Bauhinia bowkeri*), **Bag** (*Bauhinia galpinii*), **Bap** (*Bauhinia petersiana*), **Bav** (*Bauhinia variegata*), **Erl** (*Erythrina latissima*), **Inc** (*Indigofera cylindrica*), **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*) 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).

5.4.2. Effective concentration required to reduce DPPH radical (oxidant) by half (EC_{50})

The phenolic-enriched crude extracts and fractions exhibited strong radical scavenging activity against DPPH radicals in a dose dependent manner (Table 5.1). There are significant variations in the capacity of the test samples to scavenge the DPPH radical with EC_{50} ranging from 0.21±0.03 to 303.65±3.84 µg/ml. Butanol fractions of *Combretum padoides* had the highest anti DPPH radical activities compared to crude extracts and other fractions with EC_{50} 0.21±0.03 µg/ml followed by butanol fractions of *Combretum vendae* and *Combretum woodii* with EC_{50} 0.25±0.06 and 0.33±0.01 µg/ml respectively. The EC_{50} of these fractions are lower than the EC_{50} of the positive controls (trolox 1.18±0.06 – 1.31±0.07 µg/ml and ascorbic acid 1.50±0.06 – 1.68±0.07 µg/ml). As expected the non-polar compounds present in the hexane and dichloromethane fractions had poor radical scavenging activities compared to the controls.

Table 5.1. DPPH radical scavenging potential of the crude extract and fractions expressed as EC_{50} (µg/ml)

Plant species	Crude	Hexane	DCM	ETOAc	Butanol	Residual Water
Bab	19.53±4.83[a]	11.14±3.59[b]	5.21±1.04[b,c]	1.25±0.23[c]	0.64±0.05[c]	4.99±0.56[c]
Bag	14.39±0.48[a]	79.58±13.14[b]	9.92±1.16[a,c]	2.82±0.44[a,c]	1.02±0.06[c]	2.86±0.42[a,c]
Bap	43.29±5.05[a]	47.45±2.91[a]	8.18±1.11[b]	3.21±1.01[b,c]	1.51±0.07[c]	15.20±1.66[d]
Bav	123.60±11.05[a]	97.02±30.03[a]	8.40±0.62[b]	1.88±0.10[b]	0.89±0.05[b]	23.07±3.83[c]
Erl	2.54±1.40	76.71±20.25	6.02±2.0	5.61±0.37	2.24±0.05	57.98±13.94
Inc						
Scb						
Cob	5.72±1.21	85.04±10.56	20.53±0.40	7.76±0.68	4.97±0.19	35.65±4.78
Cop	4.44±0.35	12.65±1.3	3.33±0.30	0.44±0.06	0.21±0.03	0.84±0.15
Cov	1.65±0.20	16.88±2.66		1.02±0.14	0.25±0.06	0.96±0.12
Cow	3.88±1.78	9.41±3.51	2.16±0.284	1.24±0.13	0.33±0.01	1.10±0.07
Ozm	15.82±4.02	132.13±5.0	63.48±4.00	7.92±1.63	-	-
Ozp	1.29±0.07	31.95±5.6	9.77±0.71	1.22±0.44	11.79±1.12	-
Sle	1.81±0.09	54.88±2.53	10.14±1.51	0.91±0.04	38.93±0.28	-
Spd	1.19±0.15	138.5±9.50	16.51±0.77	1.26±0.03	41.8±3.37	-
Spt	4.26±0.40	139.63±10.62	4.91±0.69	2.09±0.32	-	-
Mpd	3.81±0.03	113.4±12.60	28.20±4.14	2.33±0.21	29.95±4.01	76.79±10.30
Mpr	7.39±0.32	111.2±10.69	30.80±4.56	2.52±0.30	20.71±0.90	189.50±7.56
Mse	13.46±0.52	253.0±29.69	121.46±11.0	4.73±0.06	24.01±0.13	81.17±11.39
Mun	6.99±0.14	160.4±31.4	42.88±6.16	2.23±0.15	7.86±15	303.67±3.84
Euc	3.00±0.37	134.46±10.8	4.70±0.72	0.84±0.00	0.91±0.19	2.04±0.24
Eun		84.88±9.74	7.23±4.40	1.66±0.22	1.34±0.00	2.62±1.19
Fic						
Fig						
Cam	5.85±0.55	138.46±35.73	11.03±0.77	1.85±0.27	2.55±0.23	10.4±2.36
Com	19.31±1.40	103.22±30.37	17.08±0.27	0.90±0.00	1.13±0.22	310.53±12.0
Syp						

Bab (*Bauhinia bowkeri*), Bag (*Bauhinia galpinii*), Bap (*Bauhinia petersiana*), Bav (*Bauhinia variegata*), Cam (*Carissa macrocarpa*), Cob (*Combretum bracteosum*), Cop (*Combretum padoides*), Cov (*Combretum vendae*), Cow (*Combretum woodii*), Com (*Commiphora harveyi*), Erl (*Erythrina latissima*), Euc, (*Euclea crispa*), Eun (*Euclea natalensis*), Fic (*Ficus craterestoma*), Fig (*Ficus glumosa*), Inc (*Indigofera cylindrica*), Mpd (*Maytenus peduncularis*), Mpr (*Maytenus procumbens*), Mse (*Maytenus senegalensis*), Mun (*Maytenus undata*), Ozm (*Ozoroa mucronata*), Ozp (*Ozoroa paniculosa*), Sle (*Searsia leptodictya*), Spd (*Searsia pendulina*), Spt (*Searsia pentheri*), Scb (*Schotia brachypetala*), Syp (*Syzygium paniculatum*)

Surprisingly the residual water fractions of many of the plant species also had a low antioxidant activity this may be due to the insolubility of the dried water fraction. If polyphenolics reacted with e.g. sugars to form insoluble complexes it would explain the results. The least active samples are the residual water fractions of *C. harveyi* (310.53±12.00 µg/ml) and *Maytenus undata* (303.61±3.84 µg/ml).

Other fractions with notable antioxidant activity were the butanol fractions of *Bauhinia bowkeri* (0.64±0.05 µg/ml), *Bauhinia galpinii* (1.02±0.06 µg/ml), *Bauhinia variegata* (1.51±0.07 µg/ml) and *Commiphora harveyi* (1.13±0.22

µg/ml); the ethyl acetate fractions of *C. padoides* (0.44±0.06 µg/ml), *C. vendae* (1.02±0.14 µg/ml), *Ozoroa paniculosa* (1.22±0.44 µg/ml), *Searsia leptodictya* (0.91±0.04 µg/ml), *C. harveyi* (0.90±0.00 µg/ml); and residual water fractions *C. padoides* (0.84±0.15 µg/ml), *C. vendae* (0.96±0.12 µg/ml) and *C. woodii* (1.10±0.07 µg/ml). The ethyl acetate fractions of all the 27 plant samples exhibited good antiradical activities against DPPH radical with EC_{50} ranging between 0.44±0.06 (*C. padoides*) – 7.92±1.63 µg/ml (*Ozoroa mucronata*). From the estimated EC_{50} values, the order of potency is butanol fraction > ethyl acetate fraction > crude extract > dichloromethane fraction > residual water fraction > hexane fraction.

5.4.3. Effective concentration required to reduce ABTS radical (oxidant) by half (EC_{50})

The $ABTS^{\cdot+}$ radical scavenging capacity of the crude extract and the fractions expressed as EC_{50} are presented in Table 5.2 with lower EC_{50} indicate higher antiradical activity. A wide variation in the $ABTS^{\cdot+}$ radical scavenging capacity of the crude extracts and the fractions which range from 0.43 ± 0.03 to 1709 ± 91.44 µg/ml.

The same trend of DPPH radical scavenging activity is also noticeable with the ABTS radical scavenging assay though the EC_{50} are slightly higher. Butanol fractions of *C. padoides* had the highest anti DPPH radical activities compared with crude extracts and other fractions with EC_{50} 0.21±0.03 µg/ml followed by butanol fractions of *C. vendae* and *C. woodii* with EC_{50} 0.25±0.06 and 0.33±0.01 µg/ml respectively. The EC_{50} of these fractions are notably lower than the EC_{50} of the positive controls (Trolox 1.18±0.06 – 1.31±0.07 µg/ml and ascorbic acid 1.50±0.06 – 1.68±0.07 µg/ml). Other fractions which have notable antioxidant activity were the butanol fractions of *B. bowkeri* (0.88±0.18 µg/ml), *B. galpinii* (0.89±0.04 µg/ml), *B. variegata* (1.05±0.11 µg/ml), *C. vendae* (0.60±0.03 µg/ml), *C. woodii* (0.89±0.06 µg/ml) and *E. crispa* (1.45±0.08 µg/ml).The ethyl acetate fractions of *C. padoides* (0.79±0.01 µg/ml), *C. vendae* (1.20±0.30 µg/ml), *C. woodii* (1.30±0.13 µg/ml), *S. pentheri* (1.25±0.08 µg/ml), *E. crispa* (1.34±0.03 µg/ml) including the crude extract of *O. paniculosa* (0.99±0.05 µg/ml) also have EC_{50} lower or comparable to the control. The hexane fractions exhibited poor antiradical activity with the EC_{50} being 6.78±0.29 and 1709.0±91.44 µg/ml for *C. woodii* and *C. bracteosum* respectively. From the estimated EC_{50} values, the order of potency is ethyl acetate fraction > butanol fraction > crude extract > dichloromethane fraction > residual water fraction > hexane fraction.

Table 5.2. ABTS radical scavenging potential of the crude extract and fractions expressed as EC_{50} (µg/ml)

Plant spp	Crude	Hexane	DCM	ETOAc	Butanol	Residual Water
Bab	14.50±0.50	50.63±6.37^{b}	5.54±0.48^{c}	1.81±0.13^{c}	0.88±0.18^{c}	17.99±2.34^{a}
Bag	55.01±0.25	102.25±5.04^{b}	11.81±0.90^{c}	3.21±0.22^{d}	0.89±0.04^{d}	6.21±1.03^{c}
Bap	17.19±0.14	116.27±0.57^{b}	9.76±0.20^{a}	5.01±0.07^{c}	7.31±0.85^{c}	40.44±7.09^{c}
Bav	9.24±1.30^{a}	85.84±2.55^{b}	8.58±0.43^{a}	2.40±0.22^{a}	1.05±0.11^{a}	73.91±3.68^{b}
Erl	246.37±17.73	50.89±6.08	8.76±0.22	6.52±0.19	18.52±1.10	125.00±4.22

Inc	44.95±5.60	1017.73±65.41	49.89±10.43	3.19±1.16	ND	ND
Scb	4.12±0.53	276.76±54.53	ND	5.3.3±0.60	2.09±0.45	61.64±15.23
Cob	11.34±1.5	1709±91.44	59.12±2.33	7.38±0.71	22.57±1.03	190.64±16.3
Cop	4.17±0.02	22.72±1.46	4.03±0.20	0.71±0.01	0.43±0.03	1.47±0.82
Cov	6.01±0.07	23.93±0.95	2.82±0.44	1.20±0.03	0.60±0.03	4.26±0.12
Cow	9.78±0.08	6.78±0.29	1.71±0.03	1.30±0.13	0.83±0.06	5.73±1.50
Ozm	15.93±2.10	43.48±4.20	17.55±2.79	10.74±0.45	68.85±23.76	288.17±7.05
Ozp	0.99±0.05	191.47±10.94	161.60±4.16	1.60±0.61	7.74±4.57	172.67±29.37
Sle	5.43±0.07	129.17±20.20	113.80±11.07	4.63±0.56	23.43±7.72	278.77±16.25
Spd	1.94±0.20	213.73±18.31	153.00±15.48	2.19±0.24	20.39±0.24	87.85±8.60
Spt	4.70±0.24	142.80±6.62	169.33±4.38	1.25±0.08	ND	ND
Mpd	8.64±0.13	114.64±25.93	33.54±1.29	6.33±0.18	52.79±14.43	74.89±2.80
Mpr	4.03±0.18	277.80±16.13	22.26±1.33	1.71±0.13	8.99±2.86	130.70±15.05
Mse	5.34±0.39	312.73±43.83	139.90±13.65	3.59±0.06	7.78±3.13	62.86±3.90
Mun	7.89±0.30	286.30±7.78	55.30±5.09	6.66±1.53	5.74±1.37	220.27±30.15
Euc	4.18±0.86	83.39±15.89	2.50±0.39	1.34±0.03	1.45±0.08	4.51±0.13
Eun	3.53±0.55	281.77±42.97	7.41±0.33	2.10±0.23	3.25±0.16	6.54±0.40
Fic		548.43±191.60	273.36±31.81	4.63±0.92	42.09±8.04	ND
Fig		285.43±12.83	187.53±4.54	9.06±1.51	2.80±0.50	13.93±1.02
Cam	7.87±0.93	293.97±77.70	11.68±3.66	2.66±0.14	2.45±0.21	17.92±1.37
Com	19.13±0.69	51.11±2.36	6.14±0.49	2.07±0.10	3.35±	270.03±20.94
Syp						

Bab (*Bauhinia bowkeri*), Bag (*Bauhinia galpinii*), Bap (*Bauhinia petersiana*), Bav (*Bauhinia variegata*), Cam (*Carissa macrocarpa*), Cob (*Combretum bracteosum*), Cop (*Combretum padoides*), Cov (*Combretum vendae*), Cow (*Combretum woodii*), Com (*Commiphora harveyi*), Erl (*Erythrina latissima*), Euc, (*Euclea crispa*), Eun (*Euclea natalensis*), Fic (*Ficus craterestoma*), Fig (*Ficus glumosa*), Inc (*Indigofera cylindrica*), Mpd (*Maytenus peduncularis*), Mpr (*Maytenus procumbens*), Mse (*Maytenus senegalensis*), Mun (*Maytenus undata*), Ozm (*Ozoroa mucronata*), Ozp (*Ozoroa paniculosa*), Sle (*Searsia leptodictya*), Spd (*Searsia pendulina*), Spt (*Searsia pentheri*), Scb (*Schotia brachypetala*), Syp (*Syzygium paniculatum*)

5.4.4. Ferric reducing antioxidant power (FRAP) gradient

The FRAP results are presented in Table 5.3 as the slope of the best fit linear regression analysis. Some of the ethyl acetate and butanol fractions had moderate to good dose-dependent ferric ion reducing capability comparable to the controls (trolox and ascorbic acid). The ethyl acetate fractions *Carissa macrocarpa* (45.0±3.7), *Combretumpadoides* (54.15±4.87), *Combretum vendae* (49.87±2.91), *Combretum woodii* (45.89 ± 3.87), *Commiphora harveyi* (50±3.5), *Euclea crispa* (48.0±4.8) and *Euclea natalensis* (42.0±3.9) have reducing power gradients compared to the trolox and ascorbic acid. The butanol fractions of *Bauhinia bowkeri* (40.92±2.14), *C. padoides* (44.11±4.06), *C. vendae* (42.59± 3.81), *C. woodii* (41.19 ± 1.28) and *E. crispa* (45.0±4.2) also exhibited good reducing power comparable with the control. The orders of reducing capacity is ethyl acetate > butanol > DCM > water > hexane > crude extract.

Table 5.3. Ferric reducing antioxidant power (FRAP) of the crude extracts and fractions expressed as the gradient of the linear curve

Plant species	Crude extract	Hexane fraction	DCM fraction	ETOAc fraction	Butanol fraction	Water fraction
Bab	3.30±0.17	8.15±0.76	18.75±1.18	31.84±3.36	40.92±2.14	10.26±1.21
Bag	3.63±0.19	8.36±0.41	15.20±0.59	21.48±1.55	32.70±2.10	31.75±2.08
Bap	1.51±0.05	7.57±0.34	16.42±0.83	15.50±1.69	24.19±1.22	24.63±1.14
Bav	2.63±0.27	11.74±0.62	15.22±0.75	27.75±2.58	32.06±1.11	0.47±0.05
Cam	1.70±0.10	6.1±0.57	17.0±0.87	45.0±3.7	29.1±1.7	5.2±0.59
Cob	9.4±0.32	0.27±0.037	1.6±0.10	2.7±0.40	5.2±0.25	11.0±0.37
Cop	6.36±0.26	16.96±0.91	22.68±1.04	54.15±4.87	44.11±4.06	9.59±0.58
Cov	3.310 ± 0.25	14.28±0.72	27.84±1.66	49.87±2.91	42.59±3.81	3.59±0.37
Cow	4.98±0.21	24.02±1.79	25.91±2.98	45.89±3.87	41.19±1.28	3.43±0.67
Com	1.1±0.15	14.0±0.57	26±1.7	50±3.5	36±3.0	0.26±0.08
Erl	0.29±0.07	16.0±0.60	23.0±1.3	33.0±2.9	18.0±0.84	1.8±0.13
Euc	5.5±0.17	11.0±1.2	25.0±2.1	48.0±4.8	45.0±4.2	20.0±0.91
Eun	5.3±0.27	6.1±0.18	21.0±1.2	42.0±3.9	34.0±2.2	20.0±1.9

Bab (*Bauhinia bowkeri*), Bag (*Bauhinia galpinii*), Bap (*Bauhinia petersiana*), Bav (*Bauhinia variegata*), Cam (*Carissa macrocarpa*), Cob (*Combretum bracteosum*), Cop (*Combretum padoides*), Cov (*Combretum vendae*), Cow (*Combretum woodii*), Com (*Commiphora harveyi*), Erl (*Erythrina latissima*), Euc, (*Euclea crispa*), Eun (*Euclea natalensis*)

5.4.5. Effective concentration required to reduce hydroxyl radical (oxidant) by half (EC_{50})

The scavenging ability of the crude extracts and fractions expressed as EC_{50} are presented in Table 5.4. The EC_{50} ranged from 11.03±2.80 µg/ml (dichloromethane fraction of *O. paniculosa*) to 356.80±2.39 µg/ml (dichloromethane fraction of *M. senegalensis*). The order of hydroxyl radical inhibition is butanol > ethyl acetate > crude extract > dichloromethane > hexane.

5.4. Hydroxyl radical scavenging potential of the crude extract and fraction expressed as EC_{50} (µg/ml)

Plant species	Crude	Hexane	DCM	ETOAc	Butanol	Residual Water
Mpd	23.92±2.28	110.54±17.91	122.07±20.50	70.86±18.09	49.55±5.70	
Mpr	107.69±12.32	179.70±41.17	223.96±42.04	76.70±11.56	48.79±12.42	
Mse	146.30±21.60	187.40±55.56	356.80±2.39	42.06±12.90	30.81±1.78	
Mun	80.68±2.90	284.36±27.04	311.90±150.33	30.81±1.78	51.19±5.30	
Ozm	44.29±4.20	175.56±6.88	45.77±0.98		82.24±0.97	
Ozp	33.02±6.46	35.90±3.20	11.03±2.80	33.07±0.85	17.17±3.39	
Sle	43.88±8.57	128.69±8.96	27.76±0.90	41.17±7.90	64.23±9.55	
Spd	83.46±10.45	130.45±5.70		39.02±0.90	74.51±15.46	
Spt	74.69±2.87	73.93±4.93	19.02±2.70	22.59±6.5	38.16±	

Mpd (*Maytenus peduncularis*), Mpr (*Maytenus procumbens*), Mse (*Maytenus senegalensis*), Mun (*Maytenus undata*), Ozm (*Ozoroa mucronata*), Ozp (*Ozoroa paniculosa*), Sle (*Searsia leptodictya*), Spd (*Searsia pendulina*), Spt (*Searsia pentheri*)

5.4.6. Lipid peroxidation inhibition effective concentration (EC_{50})

The inhibitory effect on the lipid peroxidation expressed as EC_{50} values are presented in Table of 5.5. The most active are *O. mucronata* and *C. woodii* with EC_{50} of 13.95±2.25 and 13.24±1.17 µg/ml respectively followed by *C. bracteosum* with 17.89±1.72 µg/ml. The least active extracts were *S. leptodictya* and *M. peduncularis* with EC_{50} of 40.45±13.38 and 39.84±5.52 µg/ml respectively.

Table 5.5: Linoleic acid peroxidation inhibition expressed as EC_{50} (µg/ml)

Plant species	EC_{50} (µg/ml)
Combretum bracteosum	17.89±1.72
Combretum padoides	35.62±4.37
Combretum vendae	30.91±2.53
Combretum woodii	13.24±1.17
Maytenus peduncularis	39. 84±5.52
Maytenus procumbens	34.21±1.63
Maytenus senegalensis	27.21±2.30
Maytenus undata	33.70±0.85
Ozoroa mucronata	13.95±2.25
Ozoroa paniculosa	25.20±8.10
Searsia leptodictya	40.45±13.38
Searsia pendulina	30.21±5.49
Searsia pentheri	25.53±6.20

5.5. Discussion

5.5.1. Qualitative antioxidant analyses (DPPH-TLC bioautography)

The antioxidant assay using DPPH on TLC plates to screen plant extracts is a quick method used to confirm the potential of the extracts for further evaluations. The intensity of the yellow spot depends on the amount and chemical characteristics of the compound present. The reaction kinetics between the DPPH radical and the active compounds varies as some compounds react slowly while others react fast. Some antioxidant spots were not readily visible immediately after sprayed with DPPH but appeared after incubation at room temperature for 2-12h. Also the mechanism of the reaction may differ as some of the compounds act as hydrogen donors and others may act as electron donors. In the DPPH radical scavenging process hydrogen donation is the predominant mechanism (Rekka and Kourounakis, 1991). This antioxidant assay is fast, simple and the image can be stored for future reference. All the extracts and fractions of the 27 plants used had antioxidant properties with varying number of yellow spots as free radical scavenging potential against the purple background of the DPPH radical on the plate. The three different mobile phases (5, 6 and 8) used to develop the chromatogram for

TLC-DPPH analyses demonstrated close relationships between the compounds present in the dichloromethane fraction of *E. latissima*, *C. vendae*, and *C. woodii*. These results indicate the danger of considering only chemical markers in taxonomy because *Combretum* and *Erythrina* are not closely related based on classical taxonomic parameters.

The antioxidant activities of the crude extracts and their fractions of varying polarities were quantified several different antioxidant assays such as $DPPH^{\cdot}$ and $ABTS^{\cdot+}$ synthetic free radicals, the hydroxyl radical and their ferric reducing capacities using the FRAP assay and lipid peroxidation inhibition. More than one type of antioxidant capacity measurement usually performed to take into account the various modes of antioxidant mechanism. These methods were not specific to any particular antioxidant component rather to the overall capacity of the extract. Of these methods only hydroxyl radical scavenging, FRAP and LPO assays have direct physiological importance as a measure of plant extracts protective performance against free radical chain reactions in cellular membranes.

For the DPPH and ABTS assay which involves hydrogen atom transfer and electron transfer processes respectively, all the extracts and fractions have a dose-dependent radical activity with butanol fraction being the most active with EC_{50} ranges from 0.21 ± 0.03 µg/ml for *Combretum padoides* to 41.8 ± 3.37 µg/ml for *Searsia pendulina* (DPPH) and EC_{50} ranges from 0.43 ± 0.03 µg/ml for *C. padoides* to 68.85 ± 23.76 µg/ml for *Ozoroa mucronata* (ABTS). These results suggested that the strong DPPH radical scavenging ability of ethyl acetate fraction was closely related to the high levels of phenolic compounds and due to the scavenging of the radical by hydrogen donation. Ethyl acetate fractions with EC_{50} ranges from 0.44 ± 0.06 µg/ml for *C. padoides* to 7.92 ± 1.63 µg/ml for *Ozoroa mucronata* (DPPH) and EC_{50} ranges from 0.71 ± 0.01 µg/ml for *C. padoides* to 10.74 ± 0.45 µg/ml for *O. mucronata* (ABTS) and the crude extracts with EC_{50} ranges from 1.91 ± 0.15 µg/ml for *S. pendulina* to 123.60 ± 11.05 µg/ml *Bauhinia variegata* (DPPH) and EC_{50} ranges from 0.99 ± 0.05 µg/ml for *Ozoroa paniculosa* to 246.37 ± 17.73 µg/ml for *Erythrina latissima* (ABTS).

In the results presented in Table 5.3, the higher the slope value the stronger the total antioxidant capacity (reduction power) of the tested extracts or fractions. The ferric reducing antioxidant power (FRAP) serve as a significant indicator of antioxidant potential of medicinal plant preparations and the activity are potentiated in the butanol and ethyl acetate fractions as observed in the DPPH and ABTS assays results. Antioxidant capacity of plant extracts and fractions depend on factors such as the compositions, chemical structures of the constituents and conditions of the test used. These results indicates that the phytochemical present in the plants performed as good electron or hydrogen donors and therefore should be able to terminate radical chain reaction by converting free radicals and reactive oxygen species to more stable products.

Ferric ions generate hydroxyl radical in vivo through Haber-Weiss and Fenton reaction mechanisms to hydroxyl. Hydroxyl radicals are highly strong reactive oxygen species, and there is no specific enzyme to defend against them in living organisms (Liu *et al*., 2005). Hydroxyl radicals and other reactive species are also produced by

activated neutrophils, eosinophils monocytes and macrophages during inflammatory responses of the immune process. Hydroxyl radicals-mediated and propagated lipid peroxidation of the gastrointestinal tract mucosa phospholipids are considered to play a crucial role in the pathophysiology of numerous chronic diseases. The major toxic products of LPO are 4-hydroxyl-2-nonenal and malondialdehyde (MDA) which can react with intestinal epithelium mucosa resulting in altered transport process (fluid and ions). These mechanisms are involved in varieties of diarrhoea aetiology such as infection, toxin, and inflammations.

ROS are important mediators that initiate and propagate inflammatory responses by inducing the formation of pro-inflammatory cytokines such as interleukin-1β (IL-1β) and tumour necrosis factors (TNF-α). ROS/RNS are generated directly by COX at the site of inflammation and have regulatory role in the expression of COX and subsequent synthesis of PGE, therefore amplifying the acute phase of the inflammatory responses. These inflammatory mediators contribute to diarrhoea aetiology as direct secretagogue (pro-secretory), reduced fluid absorption capacity due to damaged to mucosa epithelial tissue and/or modulation of the intestinal contractility through enteric nervous system (ENS).

Oxidative damage to cellular components such as cell membrane by free radicals is believed to be associated with immune system decline and hyperactivation. Immune activation of PMNs and monocytes result in formation of potent hypochlorous acid (HOCl) from myeloperoxidase (MPO)-catalyzed oxidation of Cl^- by H_2O_2. In addition, the HOCl react with primary amine groups (RNH_2) to produce N-chloramines (RNHCl). Both HOCl and RNHCl are cytotoxic (Pavlick *et al*., 2002). Some of the ROS/RSN and their products enhances intestinal and colonic Cl^- acts directly or indirectly to initiate diarrhoea as secretagogue (Gaginella *et al*., 1995).

Considerable interest is focused in finding natural antioxidants which can help on the management of numerous diseases with oxidative stress aetiology and maintenance of good health. Oxidative stress and the associated diseases resulting from an imbalance between the endogenous antioxidant defence mechanisms and pro-oxidative forces in favour can be alleviate by increased expression of antioxidant (Pavlick *et al*., 2002). Antioxidants have the capacity of stopping the chain reaction of oxidative species and the deleterious health hazard to the body. The crude extract of these medicinal plants inhibit Fenton-generated hydroxyl radical-mediated peroxidation of a heterogeneous phospholipid-aqueous phosphate buffered system and scavenged hydroxyl radical which are important characteristic of phenolic compounds. Free radical scavenging and antioxidant activity of these medicinal plants contribute their therapeutic effect against diarrhoea diseases and other GIT disorders for which they are being use ethnopharmacological in South African traditional medicine to treat.

5.6. Conclusion

In this study, the extracts and their fractions were found to have various forms of antioxidant activities that could possibly be attributed to the phenolic constituents. The extraction and fractionation protocols potentiate the

antioxidant components in the polar fractions while the non-polar hexane fractions demonstrated little or no antioxidant activity except one prominent spot from the *Erythrina latissima* extract in TLC-DPPH analyses.

Linoleic acid and arachidonic acid are indigenous compounds of the cell membrane with a task to protect the cell. The two membrane lipids are prone to attack during induce inflammatory and oxidative stress. However, the increases of intracellular ROS level, due to increased production or impaired removal, can also cause cell damage ranging from cytoplasmic swelling to cell death. In view of the involvement of the many oxidative mechanisms in the pathogenesis of various diseases, free radicals scavenging and removal of excessive ROS are important for restoring normal conditions, which might be the possible reasons of the correlation between antioxidant activity and other therapeutic activities. This investigation provided data clarifying the potentials of some of the plants as promising sources of natural antioxidants.

Further work on *in vivo* verification of the antioxidant therapeutic effectiveness, bioavailability, absorption and metabolism of the active component is needed. Finally identification, isolation, characterization and absence of possible toxicity of the bioactive compounds also required further investigation. Free radicals and oxidative species play some critical roles in diseases with inflammatory aetiologies including the GIT disorders and immunosuppression mechanism. The crude extracts and the polar fractions (ethyl acetate and butanol) of many of the plant species have strong antioxidant activities, consequently may reduce inflammation or stimulate the immune system of host. This could be one of the anti-diarrhoeal mechanisms and therefore explaining the traditional use of these medicinal plants. The anti-inflammatory potentials of the crude extracts will be investigated in the next chapter