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).

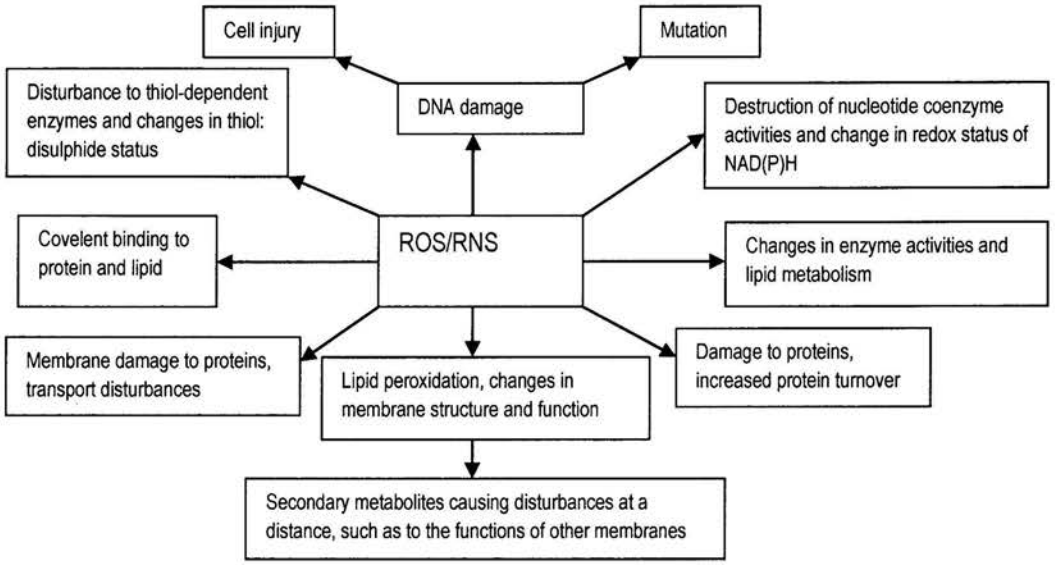

Fig. 5.1. Deleterious reactions from the production of reactive free radicals in biological system (amended from Slater *et al.*, 1987)

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) catalysed 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) catalysed 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 analyse 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 ($HO^{\cdot}$) 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 additive of absorbance 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.

ABTS (white) + $K_2S_2O_7$ (white) $\longrightarrow$ $ABTS^{\bullet+}$ (blue-green)

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.

$Fe(CN)_6^{3-}$ + antioxidant $\longrightarrow$ $Fe(CN)_6^{4-}$ + oxidized antioxidant

$Fe(CN)_6^{4-} + Fe^{3+} \longrightarrow Fe[Fe(CN)_6]^-$

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 Antioxidant assay

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. Ferric Reducing Antioxidant Power (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^{O}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 concentrations 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.

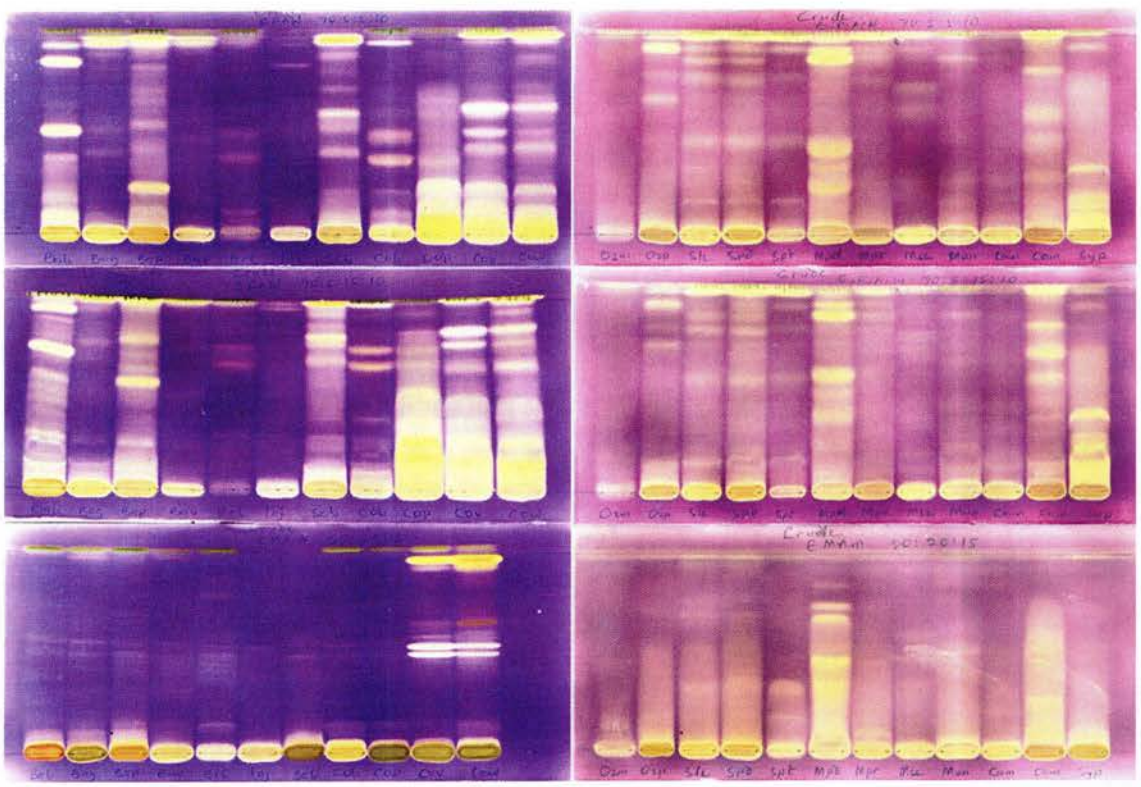

Fig.5.2. TLC-DPPH profiles of the crude extracts of extracts of different plants (left to right **Bab** (*Bauhinia bowkeri*), **Bag** (*Bauhinia galpinii*), **Bap** (*Bauhinia petersiana*), **Bav** (*Bauhinia variegata*), **Erl** (*Erythrina latissima*), **Inc** (*Indigofera cylindrica*), **Scb** (*Schotia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

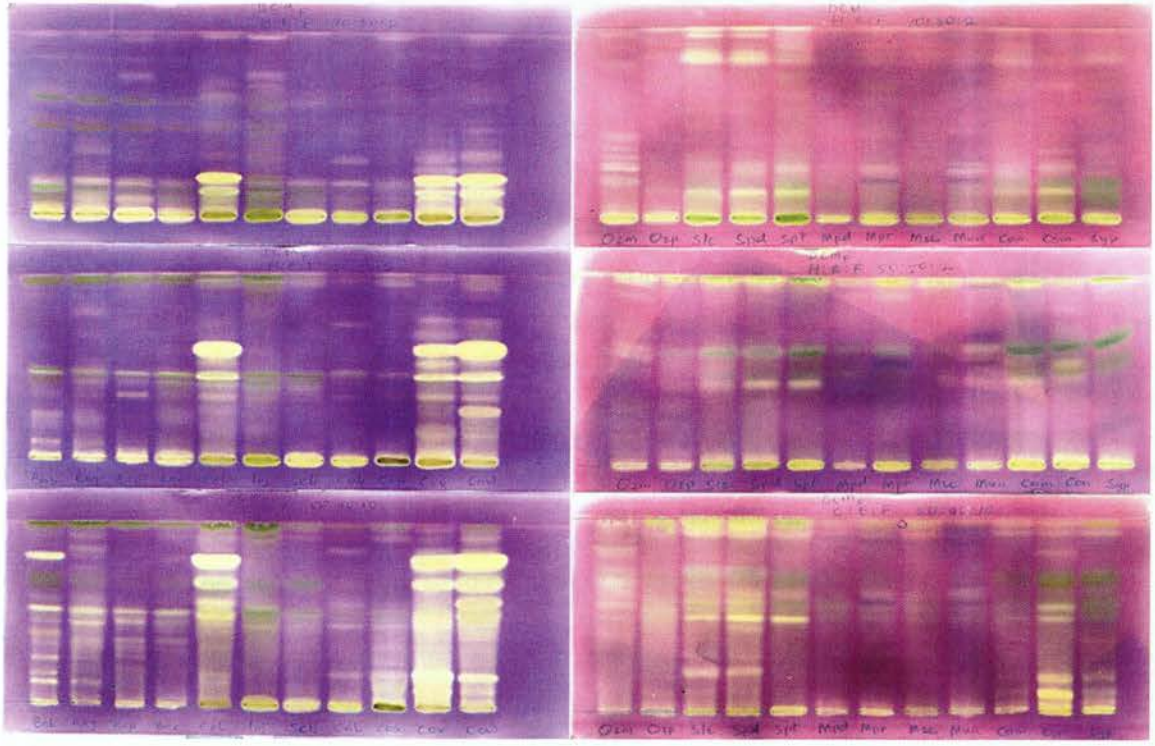

Fig.5.4 TLC-DPPH profiles of the dichloromethane fractions of different plants (left to right **Bab** (*Bauhinia bowkeri*), **Bag** (*Bauhinia galpinii*), **Bap** (*Bauhinia petersiana*), **Bav** (*Bauhinia variegata*), **Erl** (*Erythrina latissima*), **Inc** (*Indigofera cylindrica*), **Scb** (*Schotia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

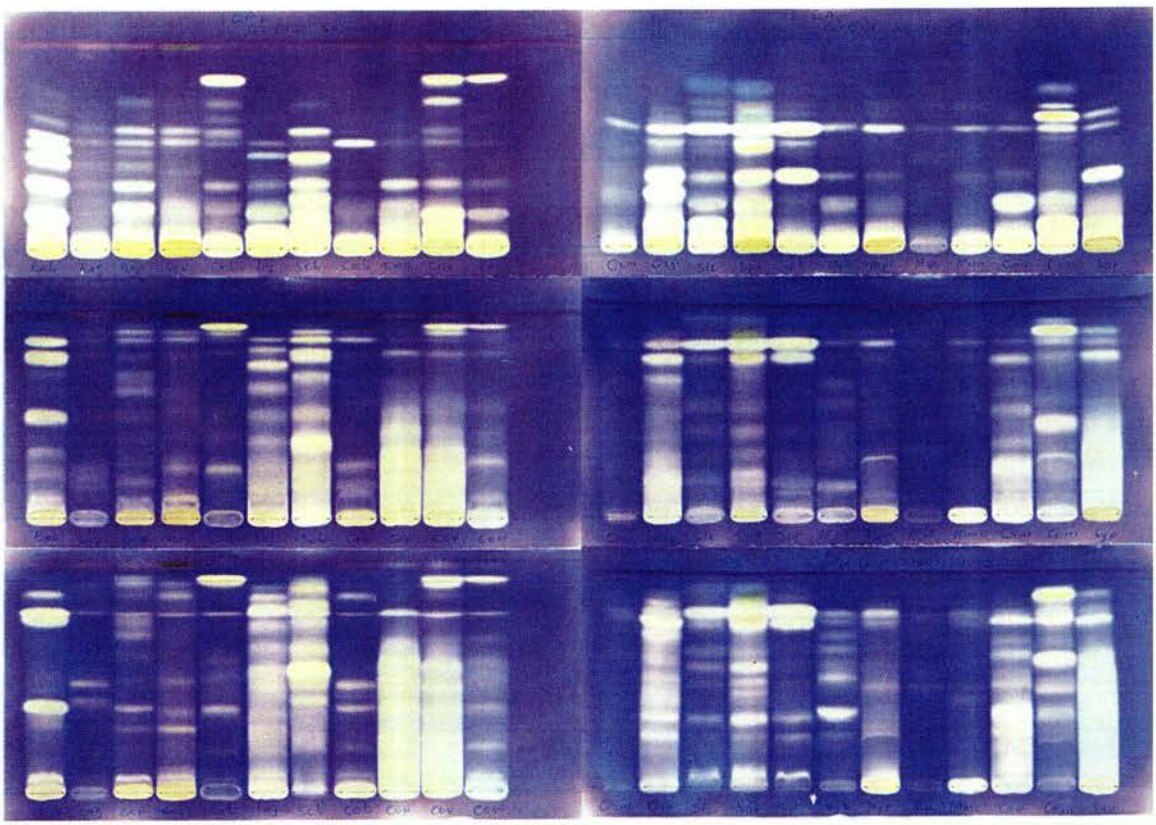

Fig. 5.5. TLC-DPPH profiles of the ethyl acetate fractions of different plants left tor right (left to right **Bab** (*Bauhinia bowkeri*), **Bag** (*Bauhinia galpinii*), **Bap** (*Bauhinia petersiana*), **Bav** (*Bauhinia variegata*), **Erl** (*Erythrina latissima*), **Inc** (*Indigofera cylindrica*), **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	11.14±3.59	5.21±1.04	1.25±0.23	0.64±0.05	4.99±0.56
Bag	14.39±0.48	79.58±13.14	9.92±1.16	2.82±0.44	1.02±0.06	2.86±0.42
Bap	43.29±5.05	47.45±2.91	8.18±1.11	3.21±1.01	1.51±0.07	15.20±1.66
Bav	123.60±11.05	97.02±30.03	8.40±0.62	1.88±0.10	0.89±0.05	23.07±3.83
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	163.83±28.80	617.30±69.13	268.46±34.25	22.33±2.54	ND	ND
Scb	25.37±7.00	290.03±156.03		15.98±6.42	6.34±5.40	17.6±3.45
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	4.10±0.31	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	ND	ND
Ozp	1.29±0.07	31.95±5.6	9.77±0.71	1.22±0.44	11.79±1.12	ND
Sle	1.81±0.09	54.88±2.53	10.14±1.51	0.91±0.04	38.93±0.28	ND
Spd	1.19±0.15	138.5±9.50	16.51±0.77	1.26±0.03	41.8±3.37	ND
Spt	4.26±0.40	139.63±10.62	4.91±0.69	2.09±0.32	ND	ND
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	6.70±0.77	84.88±9.74	7.23±4.40	1.66±0.22	1.34±0.00	2.62±1.19
Fic	13.09±2.51	388.11±42.12	295.03±10.45		5.77±0.93	51.94±2.57
Fig	11. 37±1.42	509.53±116.00	392.90±35.19	17.91±2.18	14.30±1.14	38.06±2.70
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*). ND = not determined

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 $ABTS^{\bullet+}$ 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 $ABTS^{\bullet+}$ 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	5.54±0.48	1.81±0.13	0.88±0.18	17.99±2.34
Bag	55.01±0.25	102.25±5.04	11.81±0.90	3.21±0.22	0.89±0.04	6.21±1.03
Bap	17.19±0.14	116.27±0.57	9.76±0.20	5.01±0.07	7.31±0.85	40.44±7.09
Bav	9.24±1.30	85.84±2.55	8.58±0.43	2.40±0.22	1.05±0.11	73.91±3.68
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	62.12±3.22	548.43±191.60	273.36±31.81	4.63±0.92	42.09±8.04	ND
Fig	15.21±4.21	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*). ND = not determined

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),

Combretum padoides (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 (54.74±2.13) and ascorbic acid (56.48±1.50). 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 damage 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)-catalysed 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

CHAPTER SIX

Anti-inflammatory activities of the crude extracts as antidiarrhoeal mechanisms

6.0. Introduction

Inflammation is an important component of immune response to pathogens and damaged cell characterized by heat, redness, pains, swelling and sometimes loss of tissue functionality in chronic situation (MacNaughton, 2006). Although inflammatory response provides an important defence mechanism to injurious agents, injury to some healthy cells at the inflammatory site could also occur (Sprague and Khalil, 2009).

The cellular immune systems including that of the gastrointestinal tract (GIT) act as defence mechanisms by mobilizing white blood cells (leukocytes and other chemicals) to fight infections and harmful stimuli. The body's reaction to this phenomenon may trigger inflammatory responses through the release of pro-inflammatory eicosanoids such as prostaglandins, prostacyclins and leukotrienes, and pro-inflammatory cytokines (Nardi *et al*, 2007) such as interleukins (IL-1B, IL-3,IL-6), interferons (IFN), tumor nuclear factor (TNF-α) and platelet-activating factor (PAF) (Conforti *et al*, 2008; Kunkel *et al*, 1996).

Cyclooxygenase (COX) and lipoxygenase (LOX) oxidation of polyunsaturated fatty acid (PUFA) such arachidonic acid or linoleic acid forming bioactive eicosanoids are the major features of inflammatory response (Haeggstrom *et al*., 2010) (Fig 6.1). The generation and release of reactive species (ROS/RNS) by inflammatory cells in response to pathogens and stimuli is considered the major microbicidal mechanism in the body. However, excessive generation of ROS/RNS exacerbate inflammatory responses that may lead to development of disease state.

In the GIT, inflammation affects epithelial cells as well as the more specialised mucus secreting and enteroendocrine cells of the gut mucosa (Spiller, 2004). Inflamed intestinal epithelial mucosa usually results in increased permeability, increased bowel movement or contractility, inadequate digestion of food materials and impaired absorption of essential food components (Spiller, 2004). Some inflammatory mediators are ion or fluid secretagogue and prokinetic of enteric nervous system (ENS) causing diarrhoea, and malnutrition (See section 2.10.2 for detailed discussion).

Intestinal inflammation causes damage to mucosal barrier function comprised of physical diffusion barriers, physiologic and enzymatic barriers, and immunologic barriers (Soderholm and perdue, 2006). The continuous layer of epithelial cells interconnected by tight junction, restricts both transcellular and paracellular permeation, therefore, constituting the major part of the mucosal barrier. Active secretion of fluid and mucus containing secretory immunoglobulin isotype A (IgA) also serve to bind, dilute, and cleaning mechanisms of the intestine.

The intestinal propulsive movement is also an important protective process against noxious substances (Soderholm and perdue, 2006).

6.1. Effect of cyclooxygenases (COX) on GIT

Cyclooxygenases (COXs) are oxidizing enzymes which metabolize polyunsaturated fatty acid (PUFA) such as arachidonic acid liberated from membrane phospholipid by phospholipases A to various eicosanoids such as prostaglandin D (PGD), prostaglandin E (PGE), prostacyclin (PC), thromboxane (TXA_2) (Xu *et al*, 2007; Simon *et al*, 2004). The physiological activities of these inflammatory mediators are mediated by G-protein-coupled prostanoid receptors such as DP, EP_{1-4}, FP, IP and TP which preferentially respond to PGD, PGE, $PGF_{2\alpha}$, PGI and TXA respectively. There are two isoforms of COX namely COX-1 and 2. COX-1 is constitutively expressed to produce PG series which are involve in the regulation of physiological housekeeping such as platelet aggregation, homeostasis of the GIT and the kidney. The COX-2 is an inducible enzyme expressed to produce PG series which are responsible for pro-inflammatory stimuli such as cytokines, growth factors, tumor promoting agents and bacterial endotoxins.

Prostaglandins are widely distributed along the GIT and are involved in a number of physiological and pathological processes including motility, blood flow, water and electrolyte absorption, and mucus secretion. PGE_2 is cytoprotective to the intestinal epithelium by decreasing gastric acid secretion, thus prevents ulceration. However, in pathological situation, it also increases intestinal motility and intestinal secretion causing secretory diarrhoea.

6.2. Effects of Lipoxygenase in GIT

Lipoxygenases (LOXs) comprise a family of non-heme iron-containing dioxygenases, representing the key enzymes in the biosynthesis of leukotrienes from PUFA. Leukotrienes have been postulated to play essential role in the pathophysiology of several inflammatory and allergic diseases. The LOXs are classified with respect to their positional specificity of arachidonic acid oxygenation as 5-LOX, 9-LOX, 12- LOX, 15-LOX. The products of LOXs catalysed oxygenation include leukotrienes, lipoxins, hydroperoxyeicosatetraenoic acids (HPETE), and hydroxyeicosatetraenoic acids (HETE).

LTB_4 is synthesized by 5-LOX from arachidonic acid. It is a potent chemotactic agent for inflammatory cells such as neutrophils and macrophages. It elicits leukocytes migration towards inflammatory sites and activates neutrophils, causing their degranulation associated with enzyme release as well as superoxide radicals. It also plays an important role in immune systems by enhancing the release of pro-inflammatory cytokines by macrophages and lymphocytes.

6.3. Effects of cytokines on GIT

Secretion of cytokines by the intestinal immune system is one of the main factors in maintaining the gut integrity in quiescent homeostasis. Cytokines are classified as pro-inflammatory (TNF-a, IL-1, -6, -12, -15, -18, and -32, as well as the anti-inflammatory cytokines IL-10 and TGF-β produced predominantly by activated macrophages, involved in the up-regulation of inflammatory reactions and IFN-γ and IL-4 from T-cells (MacNaughton, 2006; Sprague and Khalil, 2009). Anti-inflammatory cytokines such as IL-4, IL-10, IL-13, IFN-α, and TGF-β are involved in the down regulation of inflammatory reactions (Sprague and Khalil, 2009). Of major importance is the balance between pro-inflammatory cytokines such as TNF-α, IL-1 and IFN-γ and regulatory cytokines like IL-10 and transforming growth factor-β. The features ultimately determine the capacity of an immune response as either detrimental or innocuous to the gut. TNF-α is a critical cytokine that elaborate inflammatory responses by activating a number of inflammatory cells including neutrophils, macrophages and NK cells which induces the production of inflammatory cytokine such as IL-1β, IL-6 and IL-8 and upregulation of adhesion molecules on cell surface. In addition TNF-α also directly potentiate the immune response of other pro-inflammatory cytokines such as IL-1, IL-6, IL-12 and IFN-γ consequently enhancing the anti-inflammatory and anti-apoptotic effect. IFN-γ can stimulate the production of IL-1β, platelet-activating factor, H_2O_2, NO and downregulate IL-8. As a pro-inflammatory cytokine, IFN-γ sensitizes intestinal epithelial cells to physiological and therapeutic inducers of apoptosis.

6.4. Oxidative species as inflammatory mediator

Production of reactive species (H_2O_2, $O_2^{\bullet-}$, and $OH^{\bullet}$), nitric oxide (NO) and peroxynitrite occurs at the site of inflammation and contributes to the exacerbation of inflammatory disease and tissue damage. Oxidative species stimulates the release of pro-inflammatory cytokines such as interleukin-1β (IL-1β) and tumor necrosis factor (TNF-α). In addition, ROS induced by activated neutrophils, eosinophils, monocytes and macrophages during the inflammation process leads to tissue injury by damaging macromolecules and effecting the lipid peroxidation of membranes. In acute or chronic inflammations, the production of $O_2^{\bullet-}$ is increased at a rate that overwhelms the capacity of the endogenous SOD enzyme defence to dissipate. Reduction in the $O_2^{\bullet-}$ generation can decrease side-effects of the radical in inflammatory conditions.

Nitric oxide (NO) is a free radical gas synthesized by nitric oxide synthase (NOS) from L-arginine and initiates diverse physiological and pathological processes (Lee *et al*,, 2007b). Three iso-forms of NOS had been identified; they are neuronal NOS (nNOS), endothelial NOS (eNOS) and inducible NOS (iNOS). The first two iso-forms (nNOS and eNOS) are constitutive NOS (cNOS) while iNOS is produced only by specific stimulants in some cells. The iNOS stimulants include cytokines or bacterial lipopolysaccharides or endotoxin. Inflammatory responses are associated with the production of large quantity of NO (Cuzzocrea *et al*, 2001). The deleterious

effects of NO include mitochondrial enzymes inhibition (Nathan, 1992) and activation of COXs to produce inflammatory PGs (Salvemini and Masferrer, 1996) and interaction with superoxide to generate cytotoxic peroxynitrite. NO is an important mediator in the inflammatory process and is produced at inflamed sites by iNOS. High levels of NO have been linked to a number of pathological processes including various forms of inflammation, circulatory shock, and carcinogenesis. Therefore, an inhibitor of NOS might be effective as a therapeutic agent for inflammatory diseases.

6.5. Allopathic anti-inflammatory therapies and adverse effects on GIT

In view of the importance of PGs in inflammatory response, the rate-limiting enzymes for PGs synthesis are the therapeutic targets in controlling inflammation. Non-steroidal anti-inflammatory drugs (NSAIDs) exert their action by inhibiting the activity of COX enzymes, thereby reducing the production of pro-inflammatory prostaglandins. NSAIDs are structurally diverse, including compounds in the salicylic acid, arylalkanoic acid, propionic acid (profens), N-arylanthranilic acid (fenamic acid), pyrozolidine derivatives, oxicam and sulphonanilide families. Classical NSAIDs exhibited non-selective inhibition of both COX-1 and COX-2 while some other NSAIDs however show preferential inhibitory activity toward one isoform or the other.

Although NSAIDs provides good therapeutic relief against inflammation, some of these drugs currently in use have various side effects, particularly in the gastrointestinal tract ulceration and kidney (Charlier and Michaux, 2003). Prolonged use of non-selective NSAIDs has adverse effects such as nausea, dyspepsia, gastritis, abdominal pain, peptic ulceration, gastrointestinal bleeding, and/or perforation of gastro duodenal ulcers. In addition, NSAIDs are postulated to shift the metabolite profile from COX derivatives to lipoxygenase (LOX) derivatives resulting in the accumulation of substrate for the LOX-derived metabolites. LOX products stimulate neutrophil migration, increase adhesion of leukocytes to endothelial cells, cause smooth muscle contraction, increase vascular permeability, and increase ion and mucus secretion. Inhibition of leukotriene biosynthesis decreases inflammation and accelerates gastrointestinal healing. However, there are controversies in the recent findings that non selective NSAID (indomethacin) causes GIT damage and neither selective COX-1 inhibitors nor selective COX-2 inhibitor causes any intestinal damage. The combine use of selective COX-1 and selective COX-2 inhibitors produces intestinal haemorrhage (Takeuchi *et al.*, 2010) (Fig 6.2). Aspirin a known non selective NSAID as its metabolite such as salicylic acid causes no intestinal damage but instead provided protection against ulcerogenic response induced by other classical NSAID. This also indicates that some complex mechanisms are responsible for the intestinal damage by a number of non-selective NSAIDs other than COX-1 inhibition only (Takeuchi *et al.*, 2010). Some of the factors involved in the pathogenesis of NSAIDs toxicity include

- Bile acids secretion: NSAIDs increase the secretion of bile acids in the GIT causing complications such as colonic mucosa damage and diarrhoea. Bile acids induce the liberation of arachidonic acid from epithelial membrane, and the generation of COX and LOX metabolites along with the secondary active oxygen radicals.
- Intestinal motility: NSAIDs such as COX-1inhibitors causes marked enhancement of intestinal motility with regard to both the amplitude and frequency of contractility. Intestinal hypermotility caused mucosal hypoxia and microvascular injury due to smooth muscle contraction (Takeuchi *et al.*, 2002).
- Neutrophil infiltration: NSAIDs cause severe damage to the GIT resulting in loss of surface epithelium, mucosal necrosis and massive neutrophil infiltration.
- Bacterial flora: Non selective and COX-1 selective NSAIDs increased number of enterobacteria in the intestinal mucosa homogenates and luminal bacterial adherence to the mucosa induced severe intestinal injury (Takeuchi *et al.*, 2011).
- Nitric oxide (NO): Non selective and COX-1 selective NSAIDs cause an up-regulation of inducible NO synthases (iNOS) in the GIT. This is due to bacterial endotoxin increased intestinal permeability which induces the expression of iNOS and enhanced the generation of NO in the mucosa (Takeuchi *et al.*, 2011).
- Prostaglandins (PGs) deficiency: COX-1 isoform is expressed in most tissues, producing prostaglandins that play an important protective role in the gut by stimulating the synthesis and secretion of mucus and bicarbonate, increasing mucosal blood flow and promoting epithelial proliferation. The inhibitions of this enzyme by NSAIDs create PGs deficiency. In addition the inhibition of the COX-1 blocks platelet production of thromboxane, which increases bleeding when an active GI bleeding site is present. COX-2 isoform is induced in most tissues in response to inflammatory stimuli. Prostaglandins derived from COX-2 can be generated at the ulcer margin and appear to play an important role in ulcer healing through triggering the cell proliferation, promotion of angiogenesis and restoration of mucosal integrity (Takeuchi *et al.*, 2011). Effects of NSAIDs on GIT are presented in Fig 6.1 and Fig 6.2.

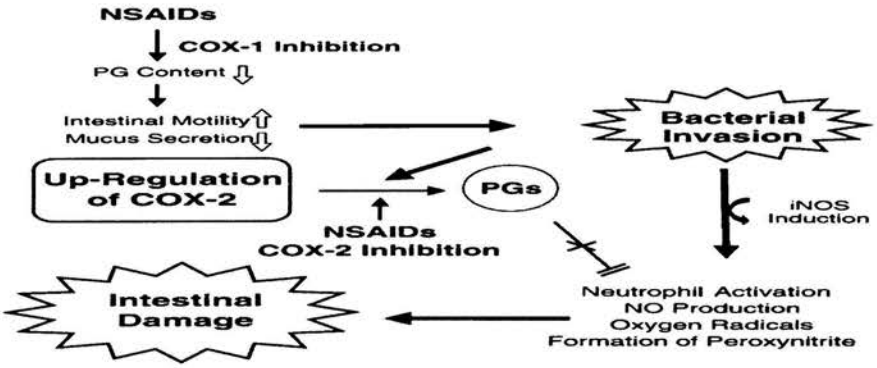

Fig. 6.1. Roles of COX-1 and COX-2 in the pathogenic mechanism of NSAID-induced intestinal damage (Takeuchi et al, 2010)

- Effect COX-2 inhibition: The NSAIDs selective inhibition of COX-2 has adverse effect on cardiovascular function (Grosser *et al*, 2006) due to suppression of PGI (anti-thrombotic) promoting hypertension and blood coagulation while the synthesis of TXA_2 (pro-thrombotic) by COX-1 remain unchanged (Fitzgerald, 2004).

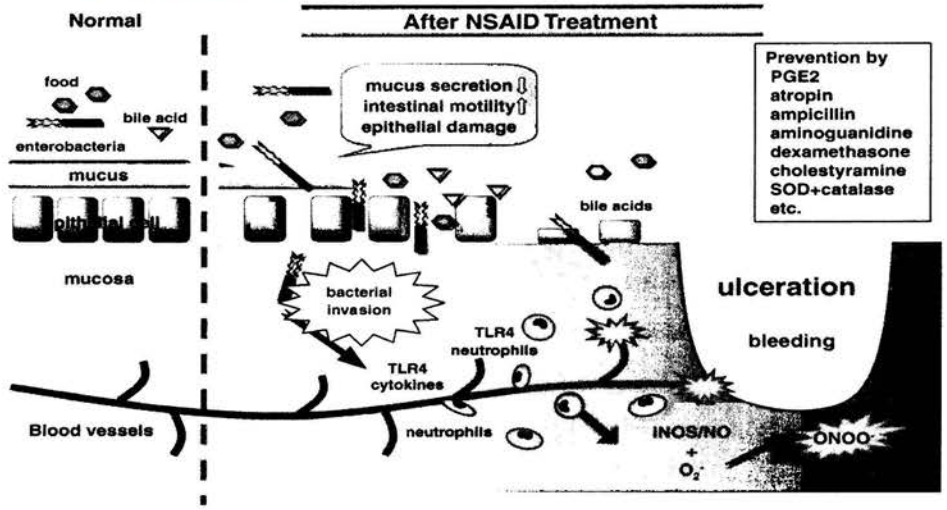

Fig. 6.2. Various factors involved in the pathogenesis of indomethacin-induced small intestinal lesions (Takeuchi et al, 2010)

6.6. Plant phytochemicals as anti-inflammatory agents

Plant extracts are suspected to contain potential bioactive component that can strongly inhibit the expression of LOX and COX. Therefore, there is continuous need to search for new drugs from natural products with anti-inflammatory properties and minimum side effects. Modulation of the activities of the enzymes implies that the inflammation process can be modified. Anti-inflammatory agent may also have an antioxidant and radical-scavenging mechanisms as part of it activity.

Phenolics, alkaloids and triterpenoids 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 potentiates the activity of SOD in radical scavenging (Nardi *et al*, 2007). Reactive species are one of the most important mediators that provokes or sustain inflammatory processes and consequently, their annihilation by antioxidants and radical scavenger such as phenolic compounds can alleviate inflammation (Delaporte *et al*, 2002; Geronikaki and Garalas, 2006).

6.7. Mechanisms of anti-inflammatory assay models

Anti-inflammatory potential were determined by in vitro assays based on the inhibitory effect on the biosynthesis of 12(S)-hydroxy-(5Z, 8E, 10E)-heptadecatrienoic acid (12-HHT), and 12(S)-hydroxy-(5Z, 8Z, 10E, 14Z)-

eicosatetraenoicacid (12-HETE). 12-HHT and 12-HETE are inflammation mediators derived from arachidonic acid metabolism, which is catalysed by enzymes of inflammatory response, cyclooxygenase (COX-1) and lipoxygenase (12-LOX), respectively. The advantage of this type of experiment is avoidance of the undesirable in vivo tests on experimental animals, since the tests commonly used to detect the anti-inflammatory activity is carrageenan induced paw oedema in rats.

6.8. Materials and Methods

6.8.1. Lipoxygenase inhibition assay

Lipoxygenase activity was determined spectrophotometrically according to Taraporewala and Kauffman, (1990); Lyckander and Malterud, (1992) which is based on the enzymatic oxidation of linoleic acid to the corresponding hydroperoxide. To determine hydroperoxide, soy lipoxygenase-1 (200 U) was incubated with linoleic acid (50 µM) in sodium borate buffer (200 mM, pH 9.0) for 4 min at 25°C. The absorbance at 234 nm was measured on a Helios β (Thermo Electron Corporation) spectrophotometer using a quartz cuvette. The inhibitory assays were performed in presence of extracts in different concentrations ranging from 0.15 - 25 µg/ml. The anti-inflammatory effect was evaluated by calculating percentage inhibition of hydroperoxide production from the changes in optical density values at 234 nm for 5 min. The test compound concentration causing 50% inhibition of hydroperoxide-release (IC_{50}) was calculated from the concentration–inhibition response curve by best fit non-linear regression analysis. The extinction coefficient of 25 mM^{-1} cm^{-1} was used for quantification of lipid hydroperoxides. DMSO was used as negative control.

6.8.2. Cyclooxygenase enzymes inhibition (COX 1 and 2) assay

The experiments were performed using an assay originally described by Noreen *et al*, (1998), with some modification by du Toit *et al*, (2005). For COX-1 assay, commercial COX-1 (from ram seminal vesicles, Sigma-Aldrich) (10 µl/sample) enzymes and Hematin (co-factor) (50 µl/sample) was pre-incubated for 5 min on ice. The mixture (enzyme and cofactor) was added to the test sample (2.5 µl of test sample and 17.5 µl of water) to make a concentration of 0.25 µg/µl in the final assay volume and pre-incubated for 5 min at room temperature. I -^{14}C-arachidonic acid (20 µl) was added to the enzyme-test sample mixture and incubated for 10 min in a water bath at 37°C. The reaction was terminated by adding 10 µl of 2M HCl. The amount of [^{14}C]-labelled PG synthesized was measured using a Packard scintillation counter after removing the unmetabolized [^{14}C]-arachidonic acid substrate by column chromatography using Pasteur pipette as column. Unmetabolized [^{14}C]-arachidonic acid substrate was eluted with n-hexane-dioxane-glacial acetic acid (70:30:1) while [^{14}C]-labelled PG synthesized in the reaction was eluted with ethyl acetate: methanol (85:15).

The same procedure was adopted for COX-2 assay using three units of COX-2 enzymes (human recombinant, Sigma-Aldrich). Indomethacin at 12.5 µM and 200 µM used as positive controls for the COX-1 assay and COX-2

assays respectively. Two background controls in which the enzymes were inactivated with HCl before the addition of [^{14}C]-arachidonic acid and two solvent blanks were prepared for experiment. The results were expressed as percentage inhibition (% I) using equation described by Lin *et al*, 1999.

% I = [(1-DPM of sample) – (DPM of background/DPM of blank - DPM of background)] X100

Where DPM is disintegration min^{-1}

6.9. Results

6.9.1. Cyclooxygenase inhibition assay

Results from cyclooxygenase assay against COX-1 are presented in Fig. 6.3. All the extracts exhibited moderate to good activity with the inhibitory effects ranging between 41.70 to 84.61%. The most active extract against COX-1 was *Carissa macrocarpa* with 82.98±1.62% inhibition at 250 µg/ml and 69.72±1.91% at 62.5 µg/ml. The inhibition of COX-1 enzyme was concentration dependent as *Bauhinia petersiana* inhibited 50% of the enzyme at a concentration of 167 µg/ml (R^2=0.989). *Bauhinia bowkeri* and *Bauhinia galpinii* inhibited 50% of the enzyme at the concentrations of 241 µg/ml (R^2=0.9645) and 377.66 µg/ml (R^2=0.9216) respectively. *Commiphora harveyi* was only active at concentration of 250 µg/ml by inhibiting 45.45±2.96% of COX-1 enzyme.

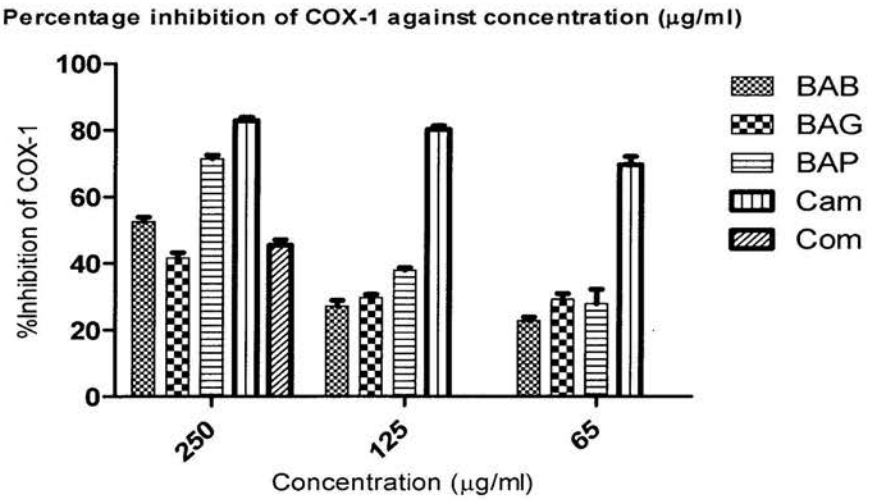

Fig. 6.3. COX-1 inhibitory activity of some selected phenolic-enriched crude extracts

The phenolic-rich crude extracts of these plants had no inhibitory activity against COX-2 at the maximum concentration tested (250 µg/ml) indicating that the extracts were COX-1 selective inhibitor. The phenolic enrich crude extracts of *C. padoides*, *C. vendae* and *C. woodii* exhibit no cyclooxygenase inhibitory activity against COX-1 and 2 enzymes. Indomethacin used as reference compound had IC_{50} of 3.30±0.006 and 122.5 µM against COX-1and COX-2 respectively.

6.9.2 Lipoxygenase inhibitory assay

The lipoxygenase inhibitory capacity expressed as LC_{50} and percentage inhibition are presented in Table 6.1. The activity of the extract varied widely ranging between 0.86±0.27 and 111.44±37.28 µg/ml. The most active extract was obtained from *Syzygium paniculatum* with LC_{50} of 0.86±0.27 µg/ml and percentage inhibition of 66.74±2.07 followed by *Euclea crispa* with LC_{50} of 2.55±0.13 µg/ml and percentage inhibition of 63.06±1.75. The least active extract was from *Commiphora harveyi* with LC_{50} of 111.44±37.28 µg/ml and percentage inhibition of 39.15±1.92 (extract concentration equals 25.6 µg/ml).

Table 6.1: Lipoxygenase inhibitory activity of the crude extracts

Plant species	LC_{50} (µg/ml)	% inhibition (25 µg/ml)
Bag	4.10±0.62	56.31±4.36
Bap	10.18±2.25	52.15±2.1
Bav	5.07±1.11	56.00±0.97
Cam	30.22±0.83	46.29±1.69
Cop	25.12±2.05	44.97±0.53
Cov	33.48±6.01	45.30±0.70
Cow	15.70±5.57	49.21±1.25
Com	111.44±37.28	39.15±1.92
Euc	2.55±0.13	63.06±1.75
Eun	17.23±0.13	50.46±1.49
Erl	7.25±1.84	55.09±1.85
Fic	8.48±4.35	53.87±2.25
Fig	5.02±1.46	66.66±3.64
Inc	7.90±1.87	55.37±4.25
Mpd	4.08±0.51	61.54±4.19
Mpr	11.08±3.50	55.80±3.61
Mse	10.88±1.92	53.18±2.31
Mun	4.68±2.44	56.50±1.81
Ozm	2.88±1.10	57.90±1.62
Ozp	27.33±9.16	46.80±2.20
Sle	11.60±2.61	54.80±2.21
Spd		
Spt	9.16±2.07	54.76±2.79
Scb	4.09±2.37	60.19
Syp	0.86±0.27	66.74±2.07

6.10. Discussion

6.10.1. Cyclooxygenase assay

COX enzymes are the rate-determining enzymes in the prostaglandin biosynthetic pathways. The modulation of the enzymes can help in anti-inflammatory treatments due to the key role of PG especially PGE_2 in the inflammatory response (Gale *et al*, 2007). ROS have been reported to have a regulatory role in the expression of COX, particularly COX-2 and subsequent synthesis of PGE_2 which is responsible for inflammation. Classification of inflammatory activity of extract based on extractants as 59% (minimum inhibition) by aqueous extracts tested at a final concentration of 250 µg per test solution and for organic extracts is 70%, when tested at a final concentration of 250 µg per test solution (Fennell *et al*., 2004). The polyphenolic-rich extracts of the plant species tested exhibited selective inhibition of COX-1. The results confirmed the postulation that most phenolic compounds like flavonoids exhibit COX-1 selective inhibitory activity and have no effect on COX-2 isoform (Kim *et al*, 2004). COX-1 is also reported to be involved in the inflammatory response and compensatory mechanisms between COX-1 and COX-2 have been demonstrated (Gale *et al*, 2007). COX-1 is the predominant isoenzyme in the normal gastrointestinal tract (Radi and Khan, 2006) and modulates neurogenic contraction (Smid and Svensson, 2009), while COX-2 expression is up-regulated during inflammation, where it modulates cholinergic contraction and small bowel motility. COX-2 mediated PGs from inflamed gastrointestinal mucosa may play a role in the chloride and fluid flux that helps flush GI bacteria.

However, the inhibition of COX-2 in the inflamed GI mucosa has been hypothesized to delay the resolution of GI injury. Since the polyphenolic-rich crude extracts of *C. macrocarpa, B. bowkeri, B. galpinii* and *B. petersiana* exhibited selective inhibition of COX-1, the use of these plants in traditional medicine as antidiarrhoea agents need to be monitored critically especially in term of dosage. The phenolic-enriched extracts of *C. padoides, C. vendae, C. woodii and Syzygium paniculatum* did not exhibit activities against COX-1 and COX-2. However, the anti-inflammatory activities of these extracts cannot be ruled out as it may involve in other inflammatory mediators. In diarrhoea disease, ROS and RNS are known to activate many pro-inflammatory cytokines (interleukins and TNFα), cell adhesions and COX enzymes. Oxidative damage exacerbates intestinal inflammatory response and causes a virulent cycle of oxidative stress, inflammation and increased mucosal permeability (Chen *et al*., 2007). Though, the phenolic-enriched extracts of the three *Combretum* species may not have a direct effect on COX enzymes, the significant free radical, ferric reducing properties and inhibition of lipid peroxidation may probably influence inflammation process.

6.10.2. Lipoxygenase assay

The anti-inflammatory activities of phenolic-enriched crude extracts on inflammatory mediators were measured against soybean lipoxygenase enzyme (Table 6.1). All medicinal extracts inhibited the lipoxygenase enzyme and

these inhibitory effects are concentration dependent. Lipoxygenases inhibition correlate to antioxidants because lipid hydroperoxide formations are usually inhibited as a result of the scavenging of lipid-oxy- or lipid-peroxy-radicals formed in the course of enzymatic peroxidation. Consequently, limiting the availability of lipid hydroperoxide substrates required for the catalytic cycle of lipoxygenase oxidative process (Cuello *et al.*, 2011)

6.11. Conclusion

Some of the crude extract exhibited selective COX-1 and LOX inhibitory activities in the *in vitro* enzymatic assays conducted in this study. The release of arachidonic acid is closely related to the cyclooxygenase (COX) and 5-lipoxygenase (LOX) enzyme systems. The ability of plant extracts, fractions and isolated pure compounds to inhibit both COX and LOX pathways of the arachidonate metabolism have been suggested to contribute to anti-inflammatory action (Middleton *et al.*, 2000). The inhibition of COX enzymes result in the shifting of arachidonic acid to the LOX pathway, which promotes gastrointestinal damage by recruiting leukocytes to the mucosal and stimulating gastric acid secretion. It is proposed that drugs that are capable of block both COX and LOX metabolic pathways (dual inhibitors) are best option in terms of NSAIDs. The dual inhibition of COX and LOX enhances their individual anti-inflammatory effects and reduce the undesirable side effects associated with NSAIDs, especially of the gastrointestinal tract (Fiorucci *et al.*, 2001). Further work on *in vivo* anti-inflammatory evaluation of the extracts in an animal model is needed to confirm the therapeutic potentials of these plant extracts. The crude *B. galpinii* had COX-1 and LOX inhibitory activity above 50% at concentration of 250 and 25 µg/ml respectively. Considering the GIT injury potential of some of the plants due to selective COX-1 inhibition, cellular toxicity will be evaluated in the next chapter.