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 per-oxy-nitrite 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 nonselective NSAIDs has adverse effects such as nausea, dyspepsia, gastritis, abdominal pain, peptic ulceration, gastrointestinal bleeding, and/or perforation of gastroduodenal 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.

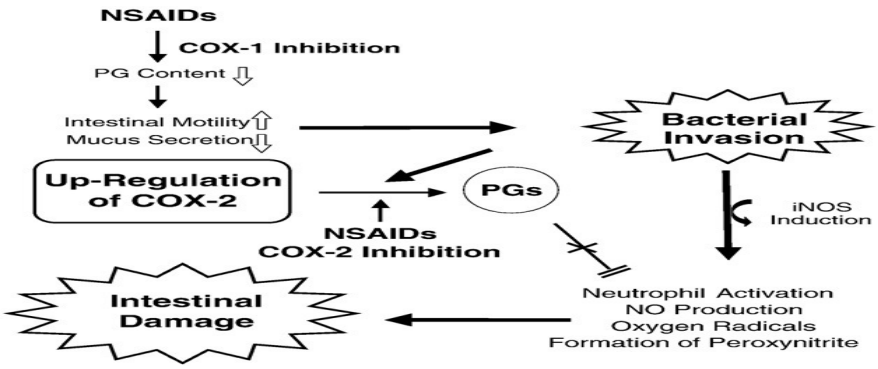

Fig. 6.1. Roles of COX-1 and COX-2 in the pathogenic mechanism of NSAID-induced intestinal damage (Takeuchi et al, 2010)

- 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).

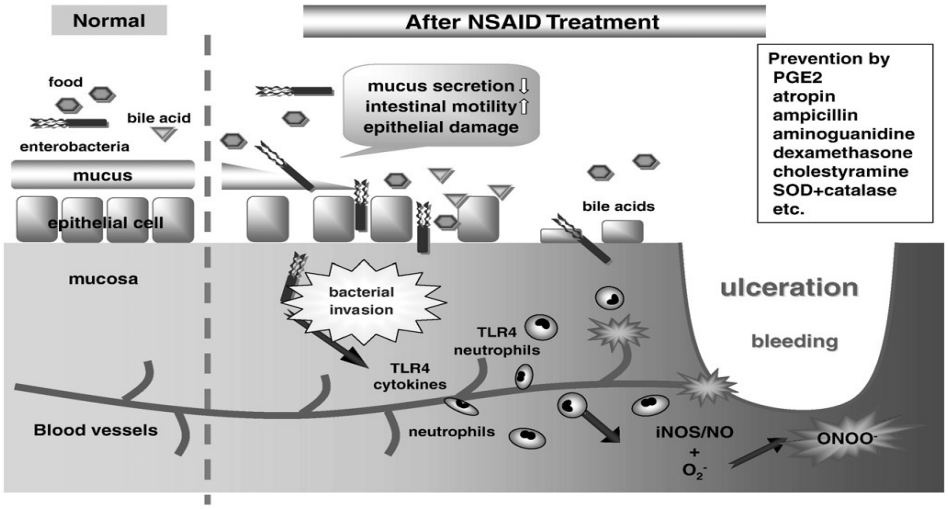

Fig. 6.2. Various factors involved in the pathogenesis of indomethacin-induced small intestinal lesions (Takeuchi et al, 2010)

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 edema 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^{o}C. The absorbance at 234 nm was measured on a Heλios 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^{0}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.

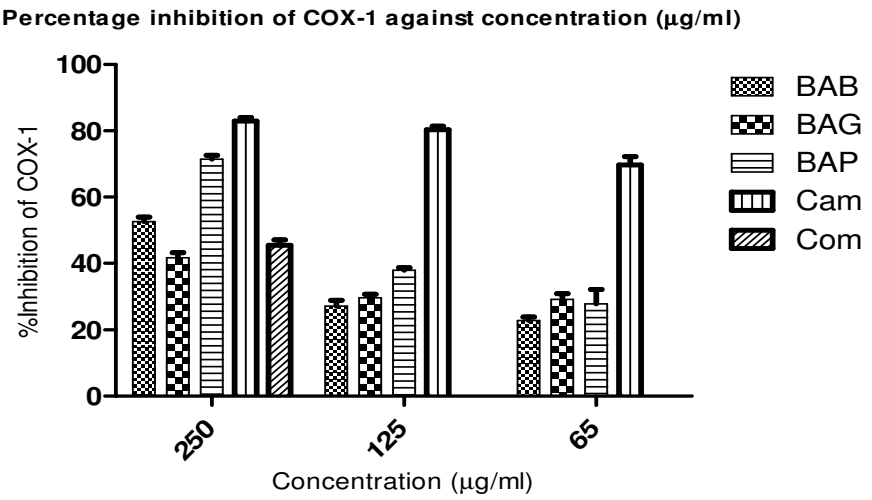

Fig. 6.3. COX-1 inhibitory activity of some selected phenolic-enriched crude extracts

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

6.9. Discussion

6.9.1. 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.

6.9.2. 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)

6.10. 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.

Chapter Seven

Cytotoxicity evaluation of the crude extracts against Vero African green monkey kidney cell lines

7.0. Introduction

Medicinal plants are assumed to be non-toxic and regarded safe due to their natural origin and long use in traditional medicine to treat various forms of diseases (Chen *et al*., 2011; Fennell *et al*., 2004). Medicinal plant preparations are administered with the hope of promoting health and treating various diseases such as infections, colds, inflammation, GIT disorders, insomnia, depression, heart diseases, diabetes, cancer, acquired immunodeficiency syndrome, and liver diseases has increased in recent times (Chen *et al*., 2011). However, scientific studies on efficacy and safety of some medicinal plants indicated that there are many phytochemicals that have cytotoxic, genotoxic, and carcinogenic effects when used chronically (Ernst, 2004; Rietjens *et al*., 2005). It should also be kept in mind that if a different extractant is used, the safety ascribed to traditional use based mainly on aqueous extracts may not be relevant at all.

The adverse effects of medicinal plant use arise due to organ toxicity, adulteration, contamination, contents of heavy metals, herb–drug interactions, poor quality control and inherent poisonous phytochemical (Jordan *et al*., 2010). Some medicinal plant phytochemicals are associated with toxicities of the heart, liver, blood, kidney, central nervous system, gastrointestinal disorder such as diarrhoea, and less frequently carcinogenesis (Jordan *et al*., 2010). In the formal herbal industry the toxicity problems of medicinal plants are attributable to insufficient quality assurance and non compliance to the standards of Good Manufacturing Practise (Palombo, 2006). Furthermore, the problem is complicated by adulteration of herbal remedies by surreptitious addition of synthetic drugs and other potentially toxic compounds such as other botanicals, microorganisms, toxins, pesticides, and fumigants (Palombo, 2006).

More importantly, if herbal medicines are used with prescription drugs especially those with narrow therapeutic indices it can result in potential harmful herb–drug interactions that cause altered drug response and toxicity (Chen *et al*., 2011). The fact that herbal medicines contain many compounds (active and non active), the large number of pharmacologically active compounds also increases the chance of herb-drug interaction (Palombo, 2006). Like synthetic drugs, herbal bioactive compounds can also undergo Phase I and Phase II enzymatic transformations to form nontoxic metabolites which are excreted through the faeces and urine. However, the production of reactive and potentially toxic metabolites is feasible with associated toxicity implications (Chen *et al*., 2011).

With the current emphasis on research and development of medicinal plant worldwide, it is important to have some information regarding the toxicity potential and efficacy of plants utilized ethnobotanically to treat ailments. As part of ethnopharmacological studies of medicinal plant available literature should be searched for known toxic properties of plants of interest before embarking on biological activity studies. However, where toxic effects are unavailable, the inclusion of cytotoxicity and other toxicity protocols in the study are useful in detecting

potential toxicity. This strategy is applicable when screening plant extracts or isolated natural products for some other biological activities such as antiinfectious, anti-inflammatory, antioxidant, antidiarrhoea and antiparasitic property. The aim of this work was to determine the potential risk of the crude phenolic-enriched extracts by evaluating the cytotoxicity using Vero cell lines.

7.1. Materials and Methods

7.1.1. Preparation of plant extract

The plant extracts were prepared as described in section 3.6.3. The dried sample were reconstituted in 70% acetone at the concentration of 1.0 mg/ml (3 ml) and from it a serial dilution of the concentration range of 1.0 to 0.001 mg/ml were made on the 96 well tissue culture plate.

7.1.2. Cytotoxicity assay against Vero cell

Cytotoxicity of the extract was determined by MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5 diphenyltetrazolium bromide] assay (Mosmann, 1983) using Vero African green monkey kidney cell lines. The cells were cultured in Minimal Essential Medium (MEM) Earle's Base, supplemented with 20 mM L-glutamine, 16.5 mM $NaHCO_3$ supplemented with 0.1% gentamicin and 5% foetal calf serum. Confluent monolayer culture suspensions of the cells were seeded into 96-well tissue culture microtitre plate at a density of 0.5×10^3 cells per well and incubated for 24 h.at 37°C in a 5.0% CO_2 incubator. The cells were washed with cultured media and extract (1.0, 0.1, 0.001 mg/ml), positive control (berberine at concentrations of 100, 10, 1.0, 0.1 µg/ml) were added and incubated for 5 days. The cells were observed using inverted microscope to check for cytopathic effect from the extract. The cells proliferation and viability was examined by addition of 30 µl of a 5 mg/ml solution of MTT in PBS to each well and incubated for another 4 h.at 37°C. The medium was carefully removed from the wells without disturbing the MTT concentrate and washed twice with PBS. The liquid was aspirated from the cells and 50 µl of DMSO was added to each well to dissolve the crystallized MTT formazan. The amount of reduced MTT was measured as absorbance at 570 nm using microtitre plate reader. The result expressed as a percentage of the control cells and IC_{50} was calculated

Dose response curves were obtained by plotting the percentage growth of cells versus log concentration of the compound. The LC_{50} (50% inhibitory concentration) values were calculated from a non-linear regression model (best fit curve) of sigmoidal dose-response curve (variable) and computed using GraphPad Prism 5.04 (Graphpad, USA).

7.2. Results

The cytotoxicity of phenolic-enriched crude leaf extracts of the 19 medicinal plants used ethnopharmacologically in treating diarrhoea and other GIT disorders is presented in Table 7.1. The results indicate that the extracts had varying degrees of toxicity to Vero cell lines with LC_{50} ranging from 3.51±2.03 to 741.90±44.22 µg/ml. The most cytotoxic extract was *Combretum woodii* (3.51±2.03µg/ml) followed by *Combretum vendae* (5.70±1.25 µg/ml)

while the least cytotoxic extract was *O. mucronata* (741.90±44.22 µg/ml) followed by *Maytenus procumbens* (187.71±19.92 µg/ml).

Table 7.1 The LD_{50} of the cytotoxicity assay of some medicinal plants used in South African traditional medicine to treat diarrhoea and related ailments

Plant species	LC_{50} (µg/ml)
Bab	17.90±2.56
Bag	35.68±2.15
Bap	40.68±18.13
Bav	76.37±7.50
Cam	ND
Cob	48.81±6.15
Cop	9.03±0.20
Cop	5.70±1.25
Cow	3.51±2.03
Com	-ND
Euc	31.61±4.04
Eun	26.99±4.48
Erl	ND
Fic	ND
Fig	ND
Inc	ND
Mpd	89.41±16.37
Mpr	187.71±19.92
Mse	87.62±3.03
Mun	99.17±11.88
Ozm	741.90±44.22
Ozp	16.58±1.85
Sle	25.09±2.40
Spd	22.30±2.42
Spt	50.62±4.30
Scb	ND
Syp	ND

ND = not determined

7.3. Discussion

For medicinal plant extracts to be useful in clinical application, the preparation must be selectively toxic to the targeted organism or interfere directly with specific reaction pathway without a major effect on the host cell or interference with normal physiological pathways. In categorization of crude extract safety, IC_{50} value of 20 µg/ml and below were considered to be cytotoxic in an in vitro assay according to US National Cancer Institute (NCI) plant screening program (Kuete *et al*., 2011) following incubation for more than 48 h. Some of the phenolic-rich crude leaf extract of the medicinal plants tested in this study are relatively toxic compared to the positive berberine control.

The cellular toxicity effects of the crude extracts were evaluated by MTT-formazan viability assay. Cellular viability and proliferation are considered to be an important functional characteristic of healthy growing cells. Increase in cell viability indicate cell proliferation, while decrease in cell viability indicate cell death as a result of either toxic effects of the test extracts or sub optimal culture conditions. With the cell viability of the negative control (DMSO) at the highest concentration of 1000 µg/ml under the same experimental condition, the latter postulate is eliminated. Therefore, all the phenolic-enriched extracts of the medicinal plants tested may be suggested to be safe for use in treating diarrhoea if the dosage is below the cytotoxic level. Although, *Ozoroa paniculosa* (16.58±1.85 µg/ml), *Searsia pendulina* (22.30±2.42 µg/ml), *Searsia leptodictya* (25.09±2.40 µg/ml) and *Euclea natalensis* (26.99±4.48 µg/ml) are within the defined cytotoxicity range, therefore the use of these extracts in traditional medicine need to be monitored carefully. It is also important to note that no report of toxicity has been recorded for the traditional use of these plant extracts. One should however remember that cellular toxicity does not necessarily equate to whole animal toxicity due to possibly interactions in the gut and bioavailability issues.

C. woodii acetone extracts have however been reported to be toxic in an *in vivo* test as anticoccidial in poultry at concentration of 160 mg/kg (Naidoo *et al*, 2008). Furthermore, several cytotoxic and anti-tumour derivative of stilbenoids such as Combretastin A and Combretastatin B5 (IC_{50} value of 10 µg/ml) have been isolated from the genus *Combretum*.

Toxicity is usually encountered due to irrational use causing accumulation of potentially toxic constituents or interactions between herbal medicinal products and conventional therapies. Indicative observations of toxicity is alterations of one of the clinical signs such as diarrhoea, weight loss, agitation, hispid hair, convulsions, tremors, dyspnoea among other) and mortality (Caparroz-Assef et al., 2005).

Some medicinal plant metabolites can cause GIT toxicity. The mechanism of action can be primarily irritative or cytotoxic in nature resulting in an initial release of mucus from goblet cells, hypersecretion from crypt cells, and maladsorption causing diarrhoea and emesis. Administration of high dose of some phytochemicals can cause effects such as necrosis, haemorrhage, and even ulceration on the GIT. Medicinal plant toxins can have additional toxicity or more directly life-threatening effects on other organ system.

7.4. Conclusion

These results are important because they show that there are risks of toxicity with an inappropriate use of some of these extracts as therapeutics for any ailments. *In vivo* acute toxicity studies may be necessary to establish the safety level of the extracts as *in vitro* assay results not necessarily translate to *in vivo* activity. Long term effect of the use of the extracts such as mutagenicity and genotoxicity also need to be determined.

In vivo animal studies are frequently very expensive and requires much work to establish changes in enzyme concentrations or histological evaluation of toxicity. It is also possible to do *ex vivo* studies using isolated organs.

In the next chapter some *ex vivo* studies will be described to investigate the possible mechanism of activity of two selected species.