Biological activities of extracts and isolated compounds from *Bauhinia galpinii* (Fabaceae) and *Combretum vendae* (Combretaceae) as potential antidiarrhoeal agents

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Declaration

The research work described in the thesis was conducted in the Phytomedicine Programme in the Department of Paraclinical Sciences, Faculty of Veterinary Science, University of Pretoria under the supervision of Professor JN. Eloff, Dr. N. Moodley, Prof. V. Naidoo and Dr. LJ. McGaw

The results presented herewith were generated from my own experiments, except where the work of others are quoted and referenced. There is no part of this work that has been submitted to any other University.

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Aroke Shahid, Ahmed

Dedication

This work is dedicated to the memory of the following: My Father (Late Mr. Ahmed Aninya Aroke), my brothers (Late Salihu Aroke and Late Ibrahim Onimisi Ahmed), Late Olukemi Ore Udom (A friend and colleague who started her PhD, but could not finish the programme before death) and my dear sister (Late Mrs. Husseinatu Ohunene Abubakar).

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Abstract

Diarrhoea is one of the killer diseases resulting from the dehydration and loss of electrolytes through profuse and excessive excretion of loose stool. The pathoaetiologies include infections, intestinal inflammation, imbalanced intestinal oxidative homeostasis and altered motility. Treatment with oral rehydration therapy (ORT) is a key intervention especially in secretory diarrhoea as supportive therapy. Symptomatic and non-symptomatic therapies directed at treating the intestinal tissues are available. However, these conventional treatments are still not sufficient in curing diarrhoea due to their associated hazards such as the development and spread of drug-resistant pathogens, changes in normal intestinal bacteria flora and potential chronic toxicity. Therapies targeted at intestinal tissue include antimotility and antisecretory agents have adverse effects such as addictiveness, constipation and fatal ischaemic colitis. Many ethnopharmacological and ethnobotanical therapies for treating diarrhoea exist among different cultures. The aims of this study were to evaluate the biological activities of plant extracts against some diarrhoeal pathophysiologies.

A literature search in English of published articles and books that discussed ethnobotanical uses of medicinal plants in southern Africa was conducted. A list of 230 medicinal plants used in South African traditional medicines for treating diarrhoea and associated complications was created. The list included family, genus, species, biological activities and bioactive isolates as well as the remedies for diarrhoea. Twenty seven species were selected to evaluate for antimicrobial, antioxidant and anti-inflammatory activities. Safety of the plants was determined by determining the cytotoxicity of the crude extracts against Vero African green monkey kidney cell lines using a standard method. Motility effects of *Bauhinia galpinii* (BGE) and *Combretum vendae* (CVE) were determined by modulation of the contractility process of the isolated rat ileum induced by spasmogens.

Phenolic compositions of the crude extract were determined using various standard methods and finally bioactivity guided isolation of antimicrobial and antioxidant compounds from BGE and CVE were carried out using open column chromatography. Identification and characterization of the isolated compounds was achieved by NMR, EI-MS and UV spectroscopy.

The non-polar fractions had good antimicrobial activities with MIC ranged between 19 – 1250 µg/ml while the polar fraction had moderate antimicrobial activities with MIC ranged between 39 - >2500 µg/ml. In general the non-polar fractions had a higher antimicrobial activity.

The crude extracts contained wide range phenolic compounds with a total phenolic (74.91±1.26 to 467.04±15.82 mg GAE/g plant material), and total flavonoids (11.27±3.37 to 176±5.96 mg EQ/g plant material). The antioxidant activities were concentrated and potentiated in the polar fractions. The non-polar fractions had poor antioxidant activities with EC_{50} values ranging from 0.21±0.03 to 303.65±3.84 µg/ml for DPPH radical scavenging and 0.43±0.03 to 1709±91.44 µg/ml for ABTS radical scavenging.

The crude extracts had selective COX-1 inhibitory activities ranging between 41.70 to 84.61% and had no COX-2 inhibitory activity. All the extracts tested had 15-LOX inhibitory capacity with LC_{50} values ranging between 0.86±0.27 and 111.44±37.28 µg/ml. The cytotoxicity results indicated a wide variation in toxic potential of the crude extracts with LC_{50} values ranging from 3.51 to 741.90µg/ml.

The BGE extracts had dual activities as spasmolytic by stimulating the spontaneous contractility and also agonised contractions induced by spasmogens but it inhibited K^+ induced contraction. CVE had spasmodic activities through a multiple mechanisms inhibiting contractions induced by spasmogens and K^+ in a dose-dependent manner.

Several bioactive xompoundswere isolated from the *Combretum vendae* leaves, There were triterpenoids (ursol-12-en-28-oic acid, mixtures of corosolic acid and maslinic acid, and asiatic acid and arjunolic acid) as well as bibenzyls combretastatin B5-O-2'-β-D-glucopyranoside, combretastatin B1-O-2'-β-D-glucopyranoside and a flavonoid (apigenin)..

From *Bauhinia galpinii* the following bioactive compounds were isolated and characterized: β-3 ethoxy sitosterol, one new flavone (5, 7, 4' 5' tetrahydroxy-2'-methoxyflavone (isoetin 2'-methyl ether) or 5, 7, 2' 5' tetrahydroxy-4'-methoxyflavone (isoetin 4'-methyl ether)), 3, 5, 7, 3', 4'-pentahydroxyflavone and 3, 5, 7, 3', 4', 5'-hexahydroxyflavone, quercetin-3-O-β-galactopyranoside and myricetin-3-O-β-galactopyranoside

The extraction protocol used in this work potentiated the antimicrobial activities in the non-polar fractions while antioxidant activities were potentiated in the polar fractions. This indicated that using polar solvents as extractant for treating infectious diarrhoea may not be quite effective unless some other antidiarrhoeal mechanisms are involved. Therefore, mixture of organic solvent (ethanol) and water can be recommended for broad-based activity.

Bauhinia galpinii extracts had a dual- mechanism of action (prokinetic and relaxant) on gastro-intestinal motility, depending on the prevalent patho-physiological condition and *Combretum vendae* mediated spasmolytic effects on isolated rat ileum through multiple inhibitions of a wide range of contractile stimuli. Hence, the presence of multiple acting spasmolytic activities in the plant extract might be contributing towards its effectiveness in treating diarrhoea and abdominal spasm. The uses of these plants in traditional medicine need to be monitored closely because of the selective inhibition of COX-1 and its associated GIT injury, and the high toxicity potential of some of the extracts.

Further work evaluating the antidiarrhoea mechanisms, identification and isolation of bioactive compounds, sub-acute and acute toxicity of the plant extracts is recommended.

Key words: Antimicrobial, antioxidant, anti-inflammatory, diarrhoeal, antispasmolytic, enteric nervous system, cytotoxicity.

List of Abbreviations

A

ABTS=2.2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid

AMP=Antimicrobial peptides

B

BAB= *Bauhinia bowkeri*

BAG= *Bauhinia galpinii*

BAP= *Bauhinia petersiana*

BAV= *Bauhinia variegata*

BGE= *Bauhinia galpinii* extract

C

Ca^{2+}= Calcium ion

Cl^-= chloride ions

CNF-1= Cytotoxic necrotising factor 1

CNS= Central nervous system

COB= *Combretum bracteosum*

COP= *Combretum padoides*

COV= *Combretum vendae*

COX= Cyclooxygenase

COW= *Combretum woodii*

CVE= *Combretum vendae* extract

D

DAEC= diffusively adherent *Escherichia coli*

DNA

DPPH=2, 2-diphenyl-1-picrylhydrazyl

E

EAEC= Enteroaggregative *Escherichia coli*

EHEC= Enterohaemorrhagic *Escherichia coli*

EIEC= Enteroinvasive *Escherichia coli*

ENS= Enteric nervous system

EPEC= Enteropathogenic *Escherichia coli*

ETEC= Enterotoxigenic *Escherichia coli*

EUC=*Euclea crispa*

EUN= *Euclea natalensis*

F

FIC= *Ficus cratestoma*

FIG=*Ficus glumosa*

FRAP= Ferric reducing antioxidant capacity

G

GIT= Gastrointestinal tract

H

HIV/AIDS= Human immune deficiency virus/Acquired immune deficiency syndrome

HOCl= hypochlorite

HUB= Haemolytic uremic syndrome

I

IBS= Irritable bowel syndrome

IL= Interleukin

INC= *Indigofera cylindrica*

iNOS= inducible nitric oxide synthase

INT= p-iodonitrotetrazolium

L

LT= Heat labile enterotoxin

LTB= Leukotriene B

M

MDA= Malondialdehyde

MCP-1= Monocyte chemoattractant protein

MIC= Minimum inhibitory concentration

MPD= *Maytenus peduncularis*

MPR= *Maytenus procumbens*

MSE= *Maytenus senegalensis*

MUN= *Maytenus undata*

N

Na^{+}= sodium ions

NAME= nitro

NH_2Cl= Ammonium chloride

NO= Nitric oxide

O

OH^{-}= Hydroxyl radical

ORT=Oral rehydration therapy

OZM= *Ozoroa mucronata*

OZP= *Ozoroa paniculosa*

P

PG= Prostaglandin

R

ROS= Reactive oxygen species

RNS= Reactive nitrogen species

S

SCB=*Schotia brachypetala*

SLE= *Searsia leptodictya*

SPD= *Searsia pendulina*

SPT= *Searsia pentheri*

ST= Heat stable enterotoxins

SYP= *Syzygium paniculatum*

T

TLC=Thin layer chromatography

TNF-α= Tumour necrosis factor-α

Trolox= 6-hydroxy-2, 5, 7, 8-tetrahydroxyl-chroman-2-carboxylic acid

U

UNICEF=United Nation Children Fund

W

WHO= World Health Organization

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CHAPTER ONE

Gastrointestinal disorders in diarrhoea diseases mechanisms and medicinal plants potentiality as therapeutic agents.

1. Introduction

The gastrointestinal tract (GIT) is dedicated to processing and absorbing nutrients and fluids essential for the maintenance of good health (Martinez-Augustin *et al.*, 2009). For the GIT to function optimally, a balance is maintained between intestinal motility and intestinal fluid volume. The latter process is finely regulated through the control of fluid absorption via intestinal villous epithelial cells and secretion across the intestine via intestinal crypt cells (Martinez-Augustin *et al.*, 2009). Net fluid absorption driven by osmotic gradients controlling the movement of electrolytes (sodium ions [Na^+] and chloride ions [Cl^-]), sugars and amino acids across the epithelial lining of the lumen, predominate in these opposing processes (Pash *et al*, 2009). In contrast, motility is controlled by the activation of enteric nervous system (ENS) by either neurotransmitters, inflammatory mediators or epithelium membrane lipid peroxidation by-products (Wood, 2004). Any upset of this delicate intestinal fluid balance (decrease fluid absorption and increase fluid secretion), and/or changes in GIT motility usually causes intestinal disorders clinically evident as diarrhoea (Vitali *et al.*, 2006).

Diarrhoea is loosely defined as an alteration in the normal bowel movement characterized by an increase in the volume, frequency and water content of stool (Baldi *et al.*, 2009). The pathophysiology of diarrhoea include microbial and parasitic infections (Hodges and Gill, 2010), stress (oxidative or physical) (Soderholm and Perdue, 2006), dysfunctional immunity (Schulzke *et al.*, 2009), disrupt GIT integrity and neurohumoral mechanisms (Vitali *et al.*, 2006; Spiller, 2004). Diarrhoea can also be a symptom of other diseases such as cholera, irritable bowel syndrome (IBS), gastroenteritis (intestinal inflammation and ulcerative colitis) (Schiller, L. R., 1999; Baldi *et al.*, 2009), malaria (Gale *et al.*, 2007) and diabetes mellitus (Forgacs and Patel, 2011).

The mechanism causing diarrhoea can be secretory (resulting from osmotic load within the intestine), hyper motility (resulting from rapid intestinal transitions) or hypo motility (resulting in decreased intestinal fluid re-absorption) or combination of these mechanisms (Vitali *et al.*, 2006). The symptoms are either caused by an increase in fluid and electrolyte secretion predominantly in the small intestine or a decrease in absorption which can involve both small and large intestine (Pash *et al*, 2009; Spiller and Garsed, 2009). Physiologically, diarrhoea is considered beneficial to the GIT as it provides an important mechanism of flushing away harmful luminal substances (Valeur *et al.*, 2009). However, diarrhoea becomes pathological when the loss of fluids and electrolytes exceeds the body's ability to replace the losses.

As a disease, diarrhoea is considered one of the most dangerous GIT disorders as death can result in severe cases due to dehydration and loss of electrolytes (WHO and UNICEF, 2004). According to the World Health Organization (WHO)/United Nation Children Fund (UNICEF) report, more than 1 billion diarrhoeal episodes occurred in human across the world yearly, with about 5 million deaths especially in infants (Thapar and Sanderson, 2004). In addition to causing acute disease and mortality, diarrhoea associated malnutrition could result in stunted growth, non-optimal immune functionality and increase susceptibility to infections. Diarrhoea therefore poses a major health challenge to human, as it could lead to premature mortality, disability and/or increase health-care costs (Guerrant *et al.*, 2005).

In animal production, diarrhoea is presumed to impose heavy productivity losses on affected farms, although true effects in monetary terms cannot be easily appreciated. The apparent on-farm losses are reduction in productivity (milk, wool, egg, meat and meat quality), increased mortality and morbidity, weight loss and abortion (Chi *et al.*, 2002). Episodes of diarrhoeal diseases can also affect the export market and hurt consumer's confidence in the products (Yarnell, 2007).

The most common modern method of managing diarrhoea is the replacement of lost fluids and electrolytes with either oral or intravenous electrolyte preparation (Thapar and Sanderson, 2004). While fluid replacement is usually effective, severe fluid losses requires additional pharmacological treatment to mitigate the on-going fluid loss. For this, drugs with antispasmodic, antimotility, antioxidative, anti-secretory/pro-absorptive and/or anti-inflammatory properties (depending on the causative agents) may be used to treat diarrhoeal (Wynn and Fougere, 2007). The issue of antimicrobial therapy for self-limiting and non-infectious diarrhoea is usually not encouraged to avoid development of drug resistance microbes. However, in cases of established infectious diarrhoea with known pathogenic agents, specific therapeutic intervention using antimicrobial drugs targeting the causative microbes may be applied. At present, the current standard therapeutic options are insufficient because of limited available modalities with broad based activities against the large number of diarrhoeal disease mechanisms and apparent side effects. The problems associated with some of the standard therapies include antimicrobial resistance, drug toxicity, constipation and addiction.

As a result there is an urgent need for new therapeutic drugs with lower cost, high efficacy, little or no side effects and wider availability especially in rural areas where diarrhoea causes large scale infant mortality. Plants, which servs as dietary source to animals and people, may also provide a good source of new therapeutic drugs.

1.2. Plant metabolites as potential therapeutic agent

Plant serves as dietary source to animals and humans providing sufficient nutrients to meet metabolic requirements for their well-being, growth and productivity. However, it can also contribute to achieving optimal

health and development as well as serving an essential role in reducing the risk or delaying the onset of diseases and disorders (Kosar *et al*, 2006; Halliwell, 1997). Medicinal plants have therapeutic properties due to biosynthesis of various complex phytochemical substances grouped broadly as phenolics, alkaloids and terpenoids. Synergistic interaction among the multiple phytochemicals may be responsible for the overall bioactivity of a given medicinal plant. Pharmacological and clinical studies of phytochemical in plants have shown that they exhibit various medicinal uses and serve as the major backbone of traditional medicine (Van Wyk and Wink, 2004). Medicinal plants have played some key roles in the health care needs of rural and urban settlements for human, livestock and animals. Plant extracts, formulations, or pure natural compounds are used in controlling diverse diseases ranging from coughs, inflammation, and diarrhoea to parasitic infection in human and veterinary medicine. A large number of these medicinal plants have been screened and validated for their ethnopharmacological use as antidiarrhoeal agents of varied mechanisms (Gutierrez *et al*., 2007). However, the literatures available on the pharmacological evaluation of medicinal plants used traditionally in treating diarrhoea in South Africa are mainly on antimicrobial screening models. Little literature information is available on other antidiarrhoeal mechanisms and *in vivo* study

For this study, 27 South African medicinal plants used as diarrhoeal remedies with ethnopharmacological background identified as requiring further biological evaluation. Thereafter, *Bauhinia galpinii* and *Combretum vendae* were choosing for further investigation based on the results from preliminary screening. *Bauhinia galpinii* was previously investigated for its antioxidant activities and three compounds (two active and one inactive) were isolated from the acetone leave extract (Aderogba *et al*., 2007). Methanol and dichloromethane leaf extracts of *B. galpinii* are reported to have antimutagenic property (Reid *et al*., 2006). The acetone root extract of *B. galpinii* has also been found to be highly cytotoxic (LD_{50} 2.70 µg/ml) against Vero cell lines (Samie *et al*., 2009). Antimicrobial activity of *Combretum vendae* against four bacterial pathogens (Ahmed *et al*., 2009) and apigenin has been isolated from the acetone leaf extract (Eloff *et al*., 2008).

In many previous studies relatively non-polar extractants were used despite the fact that traditionally aqueous extracts are used. This is probably due to difficulties in analyzing complex molecules extracted by polar extractants, because phenolics may play an important role in managing diarrhoea the focus of this study will be on more polar extractant.

1.3. Aims

To investigate the biological activities of the phenolic-enriched extracts and fractions of 27 medicinal plants against some diarrhoea pathoaetiologies and evaluating the antidiarrhoeal mechanisms of *Bauhinia galpinii* and *Combretum vendae* extracts using in vitro isolated organ methods, as means of validating their ethnopharmacological used in South African traditional medicine to treat diarrhoea.

1.4. Specific objectives

- To evaluate the effect of the extracts, fractions and isolated compound(s) against pathogenic microbes that are known to induce diarrhoea.
- To determine the antioxidative properties of the extracts, fractions and isolated compound(s) using the DPPH radical scavenging, the ABTS radical scavenging, the hydroxyl radical scavenging, the linoleic acid peroxidation inhibition and the ferric reducing antioxidant power (FRAP).
- To determine the effects of the most promising extracts on the contractility process of the isolated rat ileum induced by spasmogens, receptor agonists, antagonists and ion channels activators.
- To fractionate the extracts and elucidate the component(s) that exhibit antimicrobial and antioxidant properties.
- To evaluate the safety, efficacy and toxicity of the crude extracts and the pure active component(s).

1.5. Hypothesis

The phytochemical constituents of medicinal plants used in traditional medicine have antioxidant, anti-inflammatory, antimicrobial and /or anti-spasmodic activities that could help in alleviating diarrhoeal diseases in human and animals.

CHAPTER TWO

2.0. Literature review

2.1. Diarrhoea as a disease

Diarrhoea is a common clinical sign following on altered bowel movement, decreased intestinal absorption of fluids and increased intestinal electrolyte secretion resulting in loose and watery stool (Baldi *et al.*, 2009). The mechanisms of diarrhoea diseases can be secretory due to impaired electrolyte absorption and osmotic load within the intestine, hyper motility resulting from rapid intestinal transitions of material or hypo motility resulting in decreased intestinal fluid re-absorption or combination of these mechanisms (Vitali *et al.*, 2006). The symptoms are either caused by an increase in fluid and electrolyte secretion predominantly in the small intestine or a decrease in absorption which can involve both small and large intestine (Pash *et al*, 2009; Spiller and Garsed, 2009).

Diarrhoeal disease can be either infectious or non-infectious in nature with infection pathogenesis responsible for the major total episode worldwide. In infectious diarrhoea, the potential causative pathogens include bacterial agents (Mathabe *et al.*, 2006), rarely fungal (Robert *et al.*, 2001),, viral and parasite pathogens (Brijesh *et al.*, 2006). Non-infectious diarrhoea can be caused by adverse reactions to drugs, toxins, allergy to food, poisons and acute inflammation which promote the release of secretagogues and some enteric nervous system (ENS) receptors (prostaglandin, serotonin, substance P, vasoactive intestinal peptides, and hormone) in the GIT (Wynn and Fougere, 2007).. Diarrhoea is usually classified according to the duration of the symptoms:

- Acute diarrhoea: mostly caused by enteric pathogenic infections, intoxicants or food allergy. This type of diarrhoea is self-limiting without pharmacological intervention and usually resolves within two week from onset or,
- Persistent diarrhoea: mostly result from a secondary cause such as enteric infections or malnutrition, and usually last for more than 14 days, or
- Chronic diarrhoea: mostly result from congenital defects of digestion and absorption. This usually last for more than 30 days (Thapar and Sanderson, 2004; Baldi *et al.*, 2009).

Other methods of classifying diarrhoea include stool characteristics or pathological mechanisms such as watery, osmotic, altered motility or inflammatory diarrhoea (Ravikumara, 2008)) as shown in Fig. 2.1.

- Watery diarrhoea typically referred to as secretory diarrhoea results from increased chlorine secretion, decreased sodium absorption and increased mucosal permeability.

- Osmotic diarrhoea, also a watery form of diarrhoea, is caused by the ingestion of non-absorbable indigestible material (Baldi *et al.*, 2009) or absence of brush border enzymes required for the digestion of dietary carbohydrates (Podewils *et al*, 2004).
- Inflammatory diarrhoea is characterized by the presence of mucus, blood, and leukocytes in the stool, and is usually induced by an infectious process, allergic colitis or inflammatory bowel disease (IBD) (Ravikumara, 2008).

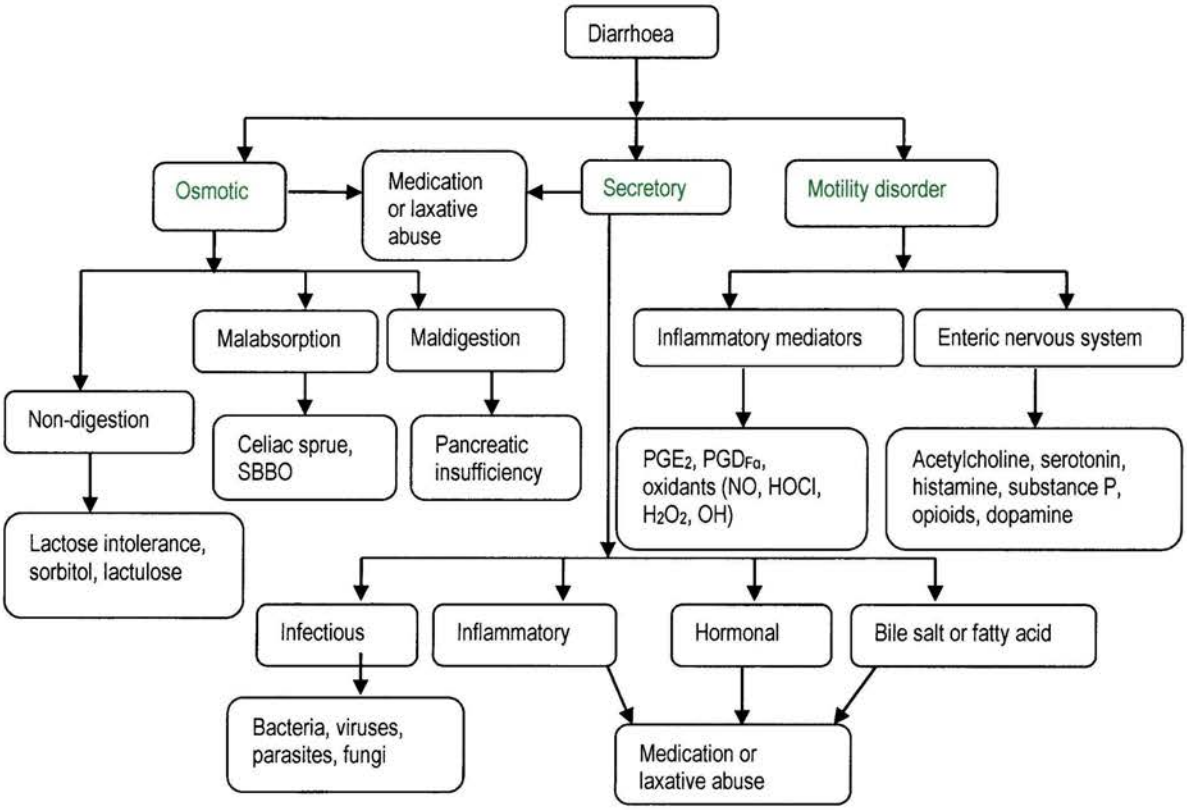


Fig.2.1: Classification of diarrhoea and the stimulants (modified from Ebert, 2005)

PGE_2= prostaglandin E_2, $PGD_{Fα}$= prostaglandin $D_{Fα}$, NO=nitric oxide, HOCl=hydrogen chlorate, H_2O_2=hydrogen peroxide, OH=hydroxyl radical, SBBO = small-bowel bacterial overgrowth

2.2. Pathophysiology of Diarrhoea

A "healthy gastrointestinal tract (GIT)" can be defined as one where a balance is reached between the bacteria colonising the environment and the immune system. Any disturbance in this homoeostasis will result in GIT disorders like diarrhoea. The general causes of diarrhoea are: microbial infection (bacteria, viruses and parasites), intestinal inflammation, altered GIT motility as a result of damage to enteric nervous system (ENS) and immune dysfunctions. The mechanisms of infectious diarrhoea include:

- Microbial attachment and localized effacement of the intestinal epithelium: Some enteric pathogens have the ability to attach and alter the surface of the invaded cell characterized as attaching and effacing lesion. Attachments of the infectious pathogens to the apical surface of the enterocyte-like cells create favourable conditions for bacterial growth and multiplication (Ramaroa and Lereclus, 2006). The mechanism of effacing lesion involves the localized destruction of the adjacent epithelial microvilli and the formation of a pedestal-like structure from the accumulation of cytoskeletal proteins, such as actin, beneath the site of attachment (Thapar and Sanderson, 2004; Guerrant *et al*, 1999).

- Production of enterotoxins that subvert mucosal transport systems: In the case of intoxication, the causative organisms may or may not be present in the transmitting medium, but act through preformed enterotoxins (examples of such organisms are *S. aureus* (α-haemolysin) *and* emetic type *Bacillus cereus* (cereulide) (Granum, 2006). The toxins may be cytotoxic or haemolytic, thus causing damage to intestinal epithelial cells. The mechanism of actions used by the enterotoxins to cause diarrhoea include (Laohachai *et al*, 2003):

(1) Decrease in intestinal surface area and, hence a decreased fluid absorption rate.
(2) Changes in mucosal osmotic permeability, resulting from mucosal destruction.
(3) Changes in fluid and electrolytes homeostasis through the toxin's action on ion channels.

- Direct epithelial cell invasion: Epithelial cells are the first major cell type encountered by infectious pathogens in the intestinal mucosa and the main site of host-pathogens interaction (Ramaroa and Lereclus, 2006). Intestinal mucosal epithelial cells are essential for initiation of the immune response to different microorganisms (Hodges and Gill, 2010). In addition to forming a physical barrier that protects the host's internal organs from the external environment, epithelial cells produce a variety of cytokines and chemokines in response to microbial infection (MacNaughton, 2006). The survival strategy of some pathogens is the invasion of epithelial mucosa cells through the activation or inhibition of different signal transduction pathways and induces cytoskeletal rearrangement within the host cell.

- Production of cytotoxins: Microbial cytotoxins degrades of the epithelial cell surface membrane and consequently results in loss of host epithelial cell layer (Ramaroa and Lereclus, 2006; Thapar and Sanderson, 2004). Diarrhoea occurs through the destruction of the epithelial cells due to loss of absorptive surface area and impaired secretion mechanisms.

- Immune activation: Some pathogens may induce diarrhoea indirectly via excessively stimulation of the immune system. The inflammatory mediators such as cytokines (interferon-γ, tumour necrosis factor (TNF-α), interleukin-6 (IL-6), and IL-1β) (Johnson *et al*., 2010), reactive oxygen species (ROS), reactive

nitrogen species (RNS) (Sprague and Khalil, 2009) can all interfere with the epithelial tight junctions (TJs), thereby resulting in diarrhoea.

- Immune inhibition: With the direct involvement of the immune system in the protection of the body against both pathogenic and enteric flora, any descent in functionality in the immune could allow the opportunity for pathogenic and/or enteric microbes to establish themselves in abundance with resultant decrease in GIT function and diarrhoea.

2.3. Detailed pathophysiology of diarrhoea

2.3.1. Inflammation in diarrhoea

Inflammation is the body's first line of defence against infection and hazardous stimuli in people and animals (Iwalewa *et al.*, 2007) with injury or infection in the GIT, resulting in the activation of neutrophils and macrophages. Once activated, the immune cell (e.g. macrophage) assist with the killing of pathogenic microorganisms and/ or the removal of harmful and cell debris (Stables and Gilroy, 2010). This task is achieved through the release of numerous pro-inflammatory cytokines (tumour necrosis factor [TNF]-α and interleukin [IL]-1β, IL-3, IL-6); chemokines and chemoattractants (IL-8 and monocyte chemoattractant protein [MCP]-1) (Conforti *et al.*, 2008) (Fig 2.2).

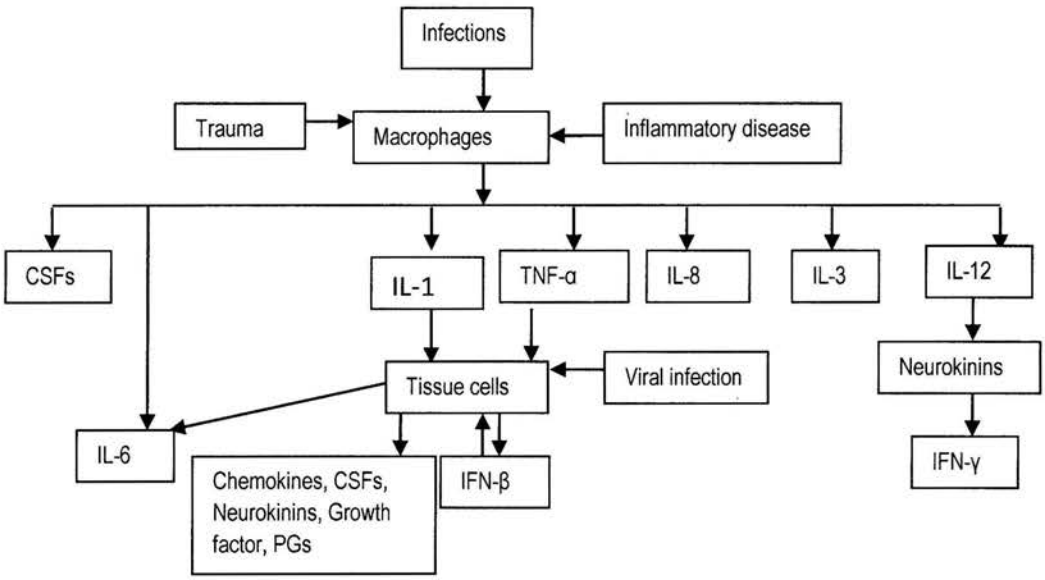


Fig. 2.2. Cytokines production network in the tissues (modified from Hopkins, 2003)

(IL=interleukin, TNF=tumour necrosis factor, CSF=colony stimulating factor, PGs=prostaglandins, IFN=interferon).

Other inflammatory nediators include ROS and RNS, eicosanoids such as cyclooxygenase products (prostaglandin E_2 (PGE_2) or lipoxygenase products (leukotrienes (LTB_4) (Nardi *et al.*, 2007) (Fig 2.3), pain

provoking mediators (histamine and bradykinin) (Matu and van Staden, 2003), and/ or cationic antimicrobial peptides (CAMP). Another antimicrobial properties of inflammation is disruption of the epithelial lining which limit microbial survival and colonization of the GIT in inflamed intestine due to loss of replication niche

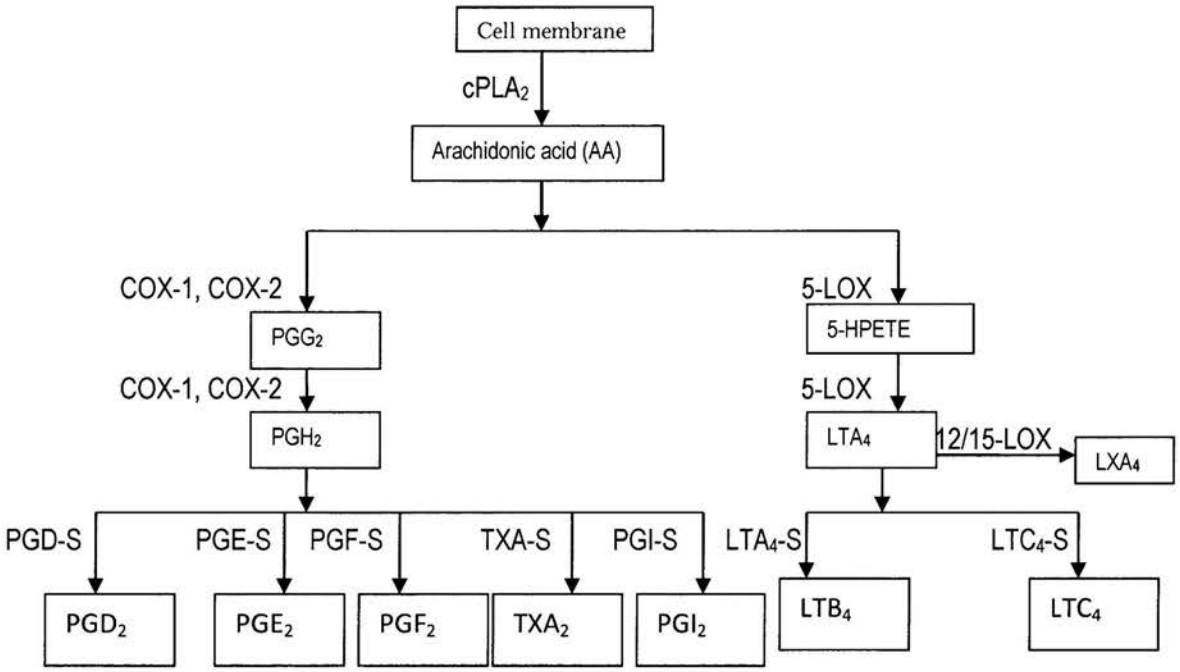


Fig.2.3. Biosynthetic pathways for the eicosanoids (modified from Haeggstrom *et al.*, 2010)
(COX=cyclooxygenase, LOX=lipoxygenase, S=synthase, PG=prostaglandin, LT= leukotriene, TXA=thromboxane, PGI=prostacyclin, $cPLA_2$=phospholipase A_2)

While inflammation process is beneficial to the body as it removes the insulting cause (Lee *et al.*, 2007a; Pharaoh *et al.*, 2006) the large recruitment and activation of neutrophil and macrophages can induced changes in gut motility, neuronal functionality, and hydro electrolyte movement with resultant diarrhoea (Gelberg, 2007). Some infectious enteric pathogens elicit inflammatory cascade and mediators to manifest diarrhoea (Guttman and Finlay, 2009).The mechanisms involved in the inflammatory modulated diarrhoea may include several factors listed below and shown in Fig 2.1.

- Epithelial barrier disruption: Gastrointestinal epithelium barrier provide a physical defence against hostile environment within the intestine lumen (Blikslager, 2010). The intestinal barrier is determined by interactions among several barrier components including the adhesive mucous gel layer, the mucosal immune system and the tight junctions (TJs) (Schenk and Mueller, 2008). The intercellular TJs are the most essential component of the intestinal physical barrier. TJs are multiple protein complexes located around the apical end of the lateral membrane of the epithelial cells. It performs dual functions as a selective/semipermeable paracellular barriers allowing movement of ion, solutes and water through the intestinal epithelium while also preventing the translocation of luminal antigens, microorganisms and

their toxins into the mucosa (Groschwitz and Hogan, 2009; Guttman and Finlay, 2009. Disruption of the intestinal TJ barrier by inflammatory cytokines, reactive oxygen species and pathogens (Guttman *et al.*, 2006) impair intestinal TJ function cause an increase in intestinal permeability resulting diarrhoea (Schenk and Mueller, 2008) as shown in Fig. 2.4.

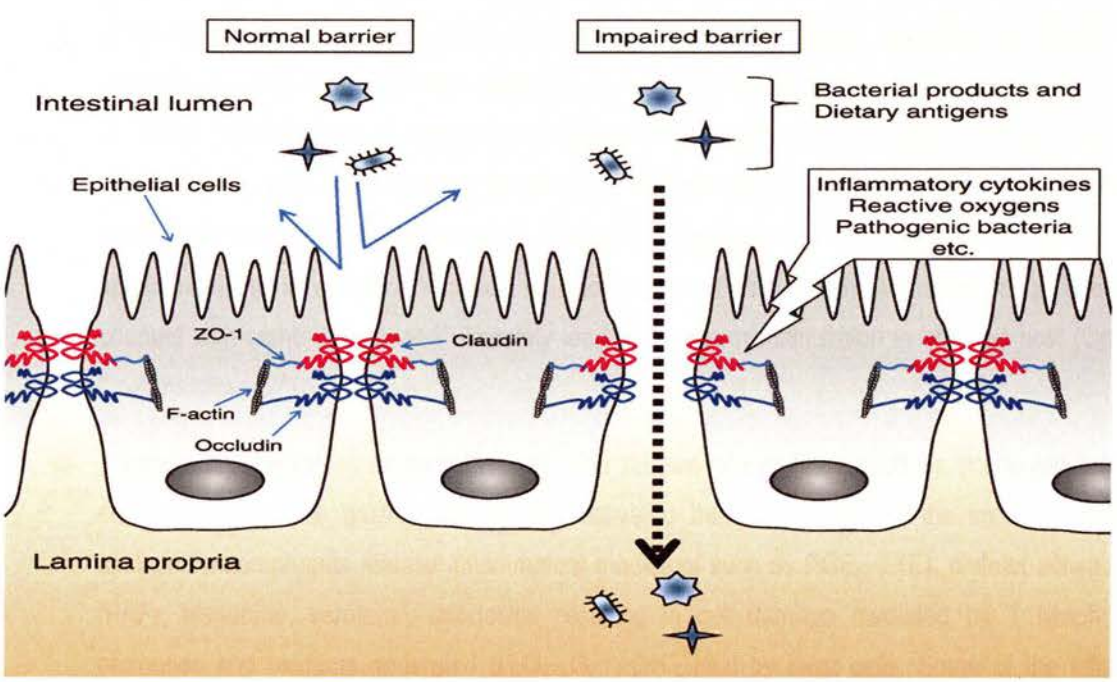


Fig. 2.4. The mechanisms of intestinal epithelial tight junctions as a physical barrier to movement of selected solute materials across the GIT. The intestinal TJs tightly regulate intestinal paracellular permeability. The barrier impairment induced by extracellular stimuli, such as inflammatory cytokines and reactive oxygens, allows the lumina bacterial products and dietary antigens to cross the epithelium and enter circulation (Suzuki and Hara, 2010).

- Reduced absorption capacity: Nutrient-coupled absorption of electrolytes takes place in the brush border microvilli (Dudeja and Ramaswamy, 2006). In an inflamed or infected intestinal tract, the total absorptive surface area is decreased due to brush border shortening resulting in malabsorption (Cotton *et al.*, 2011). Small intestinal malabsorption occurs due to impaired absorption of water, glucose and electrolytes creating an osmotic gradient that draws water into the small intestinal lumen resulting small intestinal distension and rapid peristalsis, consequently diarrhoea (Schulke *et al.*, 2009; Gelberg, 2007; Beavis and Weymouth, 1996).

- Chloride ion hypersecretion: Diarrhoeal agents such as inflammatory mediators, microbial toxins, neurotransmitters and endogenous hormones can activate inappropriate chloride ion (Cl^-) secretion from the colonic crypt epithelial cells. Excessive secretion of chloride ion (Cl^-) from the intestinal crypt is the driving force for many diarrhoea aetiologies. The underlying mechanism is the increase in intracellular levels of cyclic nucleotides (cAMP and cGMP) and/or cytosolic calcium. This process, in

turn, drives the secretion of fluid and electrolytes into the intestinal lumen, which may overwhelm the intestinal absorptive mechanism, thereby resulting in secretory diarrhoea with potential effect of severe dehydration (Petri Jr. *et al.*, 2008).

- Interference with ability to digest: Inflammatory response in the intestine may negatively affect the ability of the enterocyte to digest nutritional material. The process causes maldigestion occurs due to a deficiency in various brush border digestive enzymes, especially for carbohydrate and lipids (Schulke *et al.*, 2009). The high level of undigested carbohydrate and lipids are conversion to short chain fatty acids by the colonic microbiota and the amount may exceed colonic capacity for their absorption. Excess short chain fatty acids induced osmotic gradient pulling water and secondarily, ions into the intestinal lumen resulting in osmotic diarrhoea of colonic origin (Field, 2003). Maldigestion of ingested food coupled with osmotic diarrhoea ultimately lead to long-term malnutrition in affected host (Ogoina and Onyemelukwe, 2009).

- Inflammatory mediators as secretagogue: The release of cytokines such as interleukin-8 (IL-8) and eicosanoids into the gastrointestinal tract activated the macrophage of the immune system. The activated macrophages release inflammatory mediators such as PGE_2, LTE_4, platelet activating factor (PAF), histamine, serotonin, adenosine resulting in cell damage mediated by T lymphocytes or proteases and oxidants generated (H_2O_2, $O_2^{\cdot-}$, $OH^{\cdot}$, NO) by mast cells. Some of the inflammatory mediators (PGE_2, LTB_4, histamine) also serve as secretagogue causing secretory diarrhoea (Field, 2003).

- Stimulate enteric nervous system (ENS): Inflammation causes structural changes to the ENS that ranges from axonal damage to neuronal death (Stanzel *et al.*, 2008). The changes include altered neurotransmitters synthesis, storage and release, therefore contributing to the altered intestinal motility during the onset and progression of many GIT disorder (Stanzel *et al.*, 2008) (See section 2.3.3 for more detailed).

2.3.2. Oxidative damage in diarrhoea

Excessive generation of reactive oxygen species (ROS) or reactive nitrogen species (RNS) by the intestinal immunological system as a result of intestinal infection, irritation, inflammation, and depleted endogenous antioxidant defence causes oxidative stress (Granot and Kohen, 2003). This condition has been implicated as one of the causes of diarrhoea (Peluso *et al.*, 2002; Granot and Kohen, 2003).

The pathophysiology of oxidative stress (production) is complex and results from the normal immune response in conditions of disease (infectious and non-infectious), and is initiated by activated mitochondrial of the leukocyte.

The free radicals produced are unstable and highly reactive charged function to destroy invading organism (Dwyer *et al*., 2009). The mechanisms of ROS and RNS production involved an incomplete reduction of oxygen and nitrogen in the electron transfer chain of respiratory process in the mitochondria. In addition, immune reactions during infection or autoimmune responses through inflammation activation of a variety of inflammatory cells, which in turn activate the oxidant-generating enzymes including NADPH oxidase, inducible nitric oxide synthase (iNOS), myeloperoxidase, and eosinophil peroxidase. The ROS generated in the body are superoxide anions, hydroxyl radical, singlet oxygen and hydrogen peroxide (from leukocyte respiratory burst). The RNS included nitric oxide (NO) (produced by inducible nitric oxide synthase (iNOS). Other miscellaneous reactive species are reactive halogen and pseudohalogen species (produced by myeloperoxidase, eosinophil peroxidise, lactoperoxidase). It is well-established in vitro that free radicals may also be generated via transition metal-mediated oxidation, the so-called Fenton type chemistry, but due to the limited availability of unbound transition metals, these reactions are probably unlikely to play a major role as a source of oxidants in vivo (Chen et al., 2000).

However, since their effect is usually non-specific and aimed at the lipid membrane, the chain reaction initiated by the immune system will destroy the body's macromolecules unless scavenged (terminated). At normal physiological conditions a balance is maintained between amounts of free radicals generate and endogenous antioxidant defence system that scavenged/quenched the radicals preventing their harmful effects. Cellular antioxidant endogenous defence mechanisms are divided into three parts depending on their function:

- Quenching antioxidants: The tissue have inherent antioxidant network capable of donating electrons to oxidants, thus quenching their reactivity under controlled conditions and the derivatives become harmless to cellular macromolecules. The antioxidants however become radicals themselves, but far more stable incapable of inducing cellular damage. The oxidised antioxidants are subsequently recycled to their active reduced state by a number of efficient cellular processes fuelled by energy from NADPH. This recycling process is the main key to the efficiency of the antioxidant network.

- Repairing/removing oxidative damage: This level of antioxidant defence involves the ability to detect and repair or remove oxidised and damaged molecules before it become a threat to normal body physiological process.

- Encapsulating non-repairable damage: Finally, the body is also equipped with controlled cell suicide or apoptosis, if the extent of the oxidative damage exceeds the capacity of repair and removal.

However, a shift in favour of the radical generation, increase the burden in the body (oxidative stress) which causes tissue injury and subsequently diseases. The proposed mechanisms through which these products induced diarrhoea are presented in Fig 2.5 and discussed below:

- Lipid peroxidation are primary mechanisms for intestinal cellular malfunction, and can destroy the capacity of membranes to maintain ionic gradients resulting in an aberration in ion transport, particularly affecting potassium efflux and sodium/calcium influx (Dudeja and Ramaswamy, 2006). The production of arachidonic acid metabolites in the lipid peroxidation process can also contribute to intestinal dysfunction including diarrhoea. The ROS and RNS-induced lipid peroxidation process involves three major stages (Catala, 2009): the initiation stage, where the oxidant abstracts hydrogen from polyunsaturated fatty acids of the cell membrane, forming a radical lipid. The propagation stage may involve the rearrangement of the lipid radical to form conjugated dienes and can interact with oxygen to form lipid peroxide radicals.

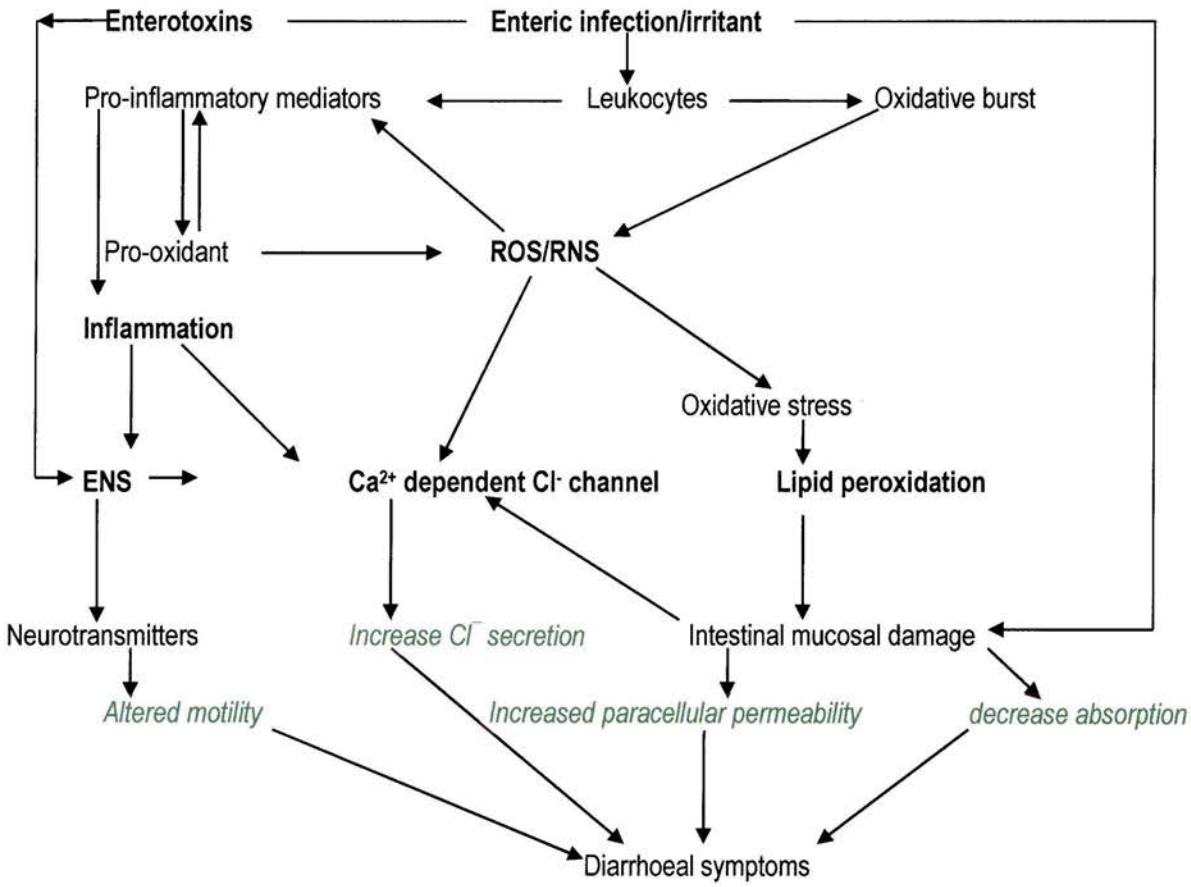


Fig.2.5 The integrative pathophysiology and mechanism of diarrhoeal disease

Bold (pathogenesis and areas of possible pharmacological intervention in diarrhoea), italic (mechanisms through which diarrhoea manifest)

The peroxide radicals can in turn abstract hydrogen from lipids to produce lipid hydroperoxide and a new radical. Lipid hydroperoxides can be oxidized, via reaction with reduced iron (Fe^{2+}) to lipid alkoxy radical and lipid peroxide, thus continuing the chain reaction of lipid peroxidation. In the final stage, the lipid peroxide radicals in the presence of reduced metals can be degraded to form highly reactive and

potent toxic aldehydes such as malondialdehyde (MDA) (Fig 2.6 and 2.7). The chain reaction can be terminated by endogenous antioxidant enzymes and exogenous antioxidant molecules by forming non-reactive substances (Catala, 2009).

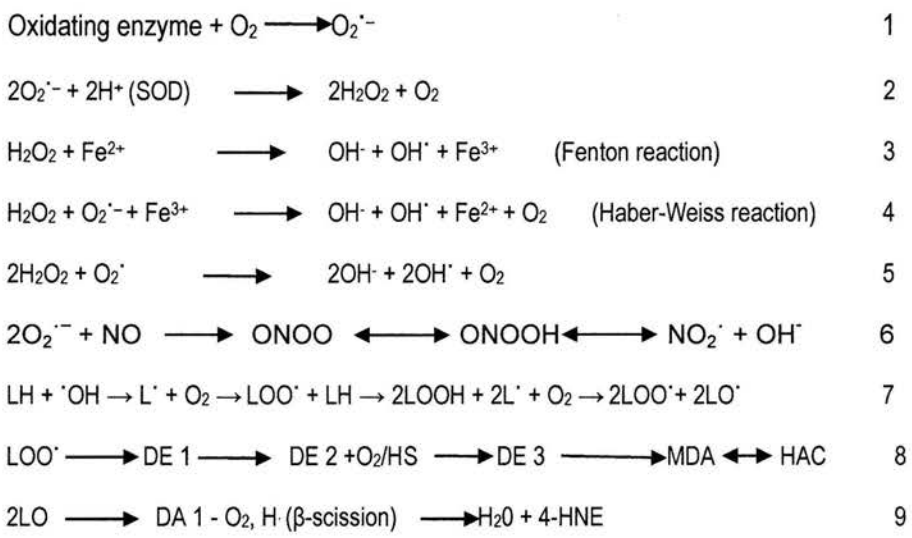


Figure 2.6: Lipid peroxidation chain reaction (Valko *et al.*, 2007).

(Equations 1 is generation of superoxide by enzymes such as NAD(P)H oxidase, xanthine oxidase and mitochondria, 2) Superoxide radical is dismutated by the superoxide dismutase (SOD) to hydrogen peroxide, 3 and 4) hydroxyl radical and hydroxyl ion hydrogen peroxide in the presence of transitional metal, 5 and 6 chain reaction to generate more radicals, 7 lipid peroxidation of phospholipids, 8 cyclization and scission of the lipid peroxide radical to generate cytotoxic malonydialdehyde (MDA), hydroxyacrolein (HAC), 4-Hydroxy nonenal (4-HNE) (Fig.2.7).

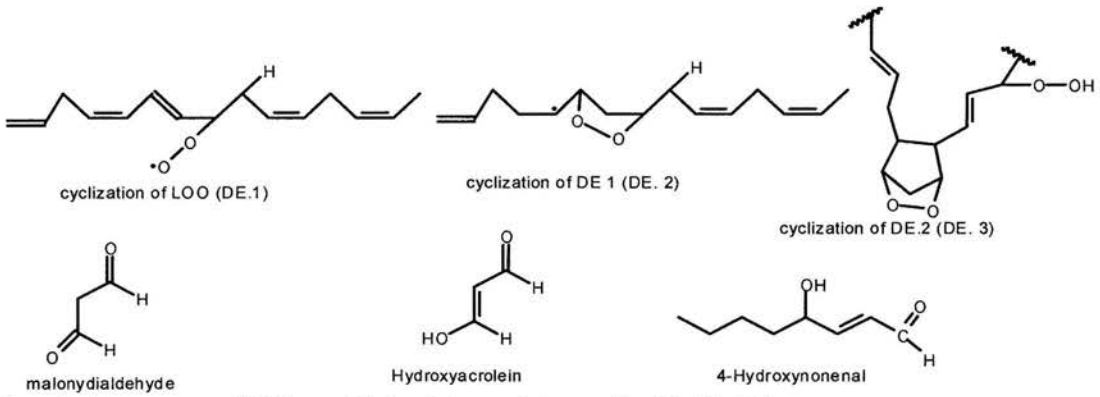


Fig.2.7. Chemical structures of lipid peroxidation intermediates outlined in Fig 2.6

- Some of the reactive species such as HOCl and NH_2Cl can also act as secretagogues on their own or can evoke the release of acetylcholine or other neurotransmitters, thus stimulating the enteric nervous system (ENS) to cause increased contractility or motility of intestinal tract (Gaginella *et al.*, 1992). The reactive species also induce gene expression by stimulating signal transduction such as Ca^{2+}-signalling and protein phosphorylation.

- Increased production of inflammatory mediators: The onset of lipid peroxidation process leads to changes in the physiological integrity of the cell membrane. The body responds to the process by 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).

2.3.3. Enteric nervous system in diarrhoea

The enteric neural network is responsible for the control of propulsive transport and segmental peristalsis in the GIT, as well as secretion and absorption across the intestinal lumen (Wood, 2004; Bohn and Raehal, 2006). While enteric nervous system (ENS) functions independently of the central nervous system (CNS), it is modulated by the parasympathetic and sympathetic autonomic nervous system (Farthing, 2003). As a unit, the ENS is a complicated physiological with autoregulation being mediated by a number of neurotransmitters such as acetylcholine, serotonin, substance P, histamine and endorphin (Farthing, 2002). Diarrhoea can result from the alteration of these systems:

- Smooth muscle contractility: Many agonists and/or antagonists elicit contractility in GIT smooth muscle (longitudinal or circular) through activation of various receptors located within the muscle (Holzer, 2004). In some cases the activation of the smooth muscle receptors by neurotransmitters and inflammatory mediators include reactive oxygen species causes relaxation (spasmolytic). While in other cases, the process lead to increase in spontaneous or induced contraction (spasmogenic). Ionic channel (Ca^{2+} and Cl^{-}) are also known to play important roles in smooth muscle contraction (Giorgio *et al*., 2007). Anion and fluid secretion into the intestine lumen are stimulated through activation of the receptors on enteric secretomotor pathways and epithelial cells, consequently causing secretory diarrhoea.

- Motility: Intestinal motility dysfunctions include situations in which movement of material along the GIT is repetitive and rapid (diarrhoea) and/or too slow (pseudo-obstruction, slow transit constipation) (Talley, 2006; Giorgio *et al*., 2007) are controlled by activities of neurotransmitters on the ENS. Pathogenic bacterial overgrowth is common as a result of intestinal hypomotility or low transit time which may lead to mucosal inflammation, increased accumulation and absorption of toxins which are known pathophysiology of diarrhoea. The mechanisms may include impaired digestion as in the deconjugation of bile salts with subsequent fat malabsorption, leading to fatty acid diarrhoea or osmotic effects of malabsorption of sugars resulting in osmotic diarrhoea. Diarrhoea also results from an increase in the gut motility (hypermotility) inducing an accelerated transit of food intake. The net fluid absorption from

the food intake is reduced due to less adequate contact time with the GIT epithelial lining for the absorption of fluids before excretion.

2.3.4. Cystic fibrosis transmembrane conductance regulator (CFTR) regulation

Cystic fibrosis transmembrane conductance regulator (CFTR) is a cyclic adenylate monophosphate (cAMP)-activated Cl^- channel expressed in epithelial cells in the intestine and other fluid-transporting tissues (Thiagarajah and Verkman, 2003). Diarrhoeal pathogens and their toxins can induce secretory diarrhoea by simultaneously stimulation of the active secretion of Cl^- and inhibition of Na^+ absorption across the apical membrane of enterocyte with resulting massive fluid and electrolyte loss into GIT (Schuier *et al.*, 2005). The cellular signalling mechanisms include an increase in cellular cAMP and cyclic guanylate monophosphate (cGMP), which may result in activation of the CFTR Cl^- channel. Pharmacological blocking of CFTR with drugs such as glibenclamide and $CFTR_{inh}$-172 inhibits salt and water loss in diarrhoea (Schuier *et al.*, 2005).

2.4. Specific Agents of Diarrhoea

2.4.1. Bacterial causes of diarrhoea

2.4.1.1. E*scherichia coli*

E. *coli* is a gram-negative rod shaped bacteria that shares a symbiotic relationship with animal host as part of normal digestive intestinal flora. Under certain define conditions these organisms or pathogenic strains of these organisms are known to induce diarrhoea (Clarke, 2001; Le Bouguenec, 2005). There are six main types of pathogenic *E. coli* associated with diarrhoea, namely enterotoxigenic *E. coli* (ETEC), enteroinvasive *E .coli* (EIEC), enteropathogenic *E. coli* (EPEC), enterohaemorrhagic *E. coli* (EHEC), enteroaggregative *E. coli* (EAEC) and diffusively adherent *E. coli* (DAEC) (Clarke, 2001). While the exact process by which each type of these *E. coli* induces diarrhoea symptoms varies significantly, the basic pathophysiology involves their inherent ability to adhere to epithelial cells and colonize the host tissues (Le Bouguenec, 2005). The characteristics and mode of actions of each type of the pathological strains in diarrhoea diseases are listed in Table 2.1. Infections from some of the strains of *E. coli* are self-limiting and can resolve without pharmacological intervention. However, symptomatic, supportive and antibiotic, or a combination of the therapies may be beneficial in the chemotherapeutic management of some cases involving ETEC, EIEC and EPEC infection (Elsinghorst, 2002). The use of antibiotics recommended, antimicrobial chemotherapeutic agents such as tetracycline, doxycycline, and ciprofloxacin may be used (Casburn-Jones and Farthing, 2004; Elsinghorst, 2002) in infectious diarrhoea.

Table 2.1: The mechanism of actions and symptoms of enteric pathogenic *E. coli* (Thapar and Sanderson, 2004; Clarke, 2001).

Strain	Mechanism of action	Symptoms	Treatments
Enterotoxigenic *E. coli* (ETEC)	Colonization of the small bowel mucosa, followed by elaboration of heat-labile (LT) and heat stable (ST) enterotoxins. The ST enterotoxins are classified as STa and STb. The binding of STa to guanylate cyclase C receptor results in increased intracellular cyclic guanylate monophosphate (cGMP) level. The resultant effect is the stimulation of chloride secretion or inhibition of sodium chloride absorption causing intestinal fluid secretion. LT enterotoxins consist of two serotypes (LT-I and LT-II). LT activate adenylate cyclase causing intracellular increase in cyclic adenosine monophosphate (cAMP) levels resulting in decrease sodium absorption by villous cells and subsequent active chloride secretion by crypt cells thus leading to osmotic diarrhoea.	Watery diarrhoea ranging from mild, self-limiting disease to severe purging.	Supportive therapy with antibiotic in cases of severe infection.
Enteroinvasive *E. coli* (EIEC)	Invasion of the epithelium and mucosal destruction eliciting inflammatory response accompanied by necrosis and ulceration of the large bowel with resultant release of blood and mucosa into the stool.	Bloody, mucoid diarrhoea and dysentery	Antibiotic in cases of bloody diarrhoea
Enterohaemorrhagic *E. coli* (EHEC)	Adhesion followed by liberation of a potent toxin which is cytotoxic to Vero cells (referred to as shiga-like cytotoxin I and II). Other mechanism attributed to the EHEC virulence includes adhesin	Bloody diarrhoea, fever, vomiting, haemorrhagic colitis, haemolytic uremic syndrome (HUB), acute renal failure, haemolytic anaemia,	Symptomatic therapy
Enteropathogenic *E. coli* (EPEC)	Adherence of the bacterium to the gut epithelium causing attachment and effacement lesion on intestinal epithelial cells, alteration of intracellular calcium and cytoskeleton.	Self-limiting watery diarrhoea with fever and vomiting	Antibiotic in severe cases
Enteroaggregative *E. coli* (EAEC)	Aggregating pattern of adherence to intestinal mucosa produces enteroaggregative heat-stable (EAST) enterotoxins causing cellular damage and function similar to, but distinct from ST enterotoxins.	Watery mucoid diarrhoea	Antibiotic in severe cases
Diffusively adherent *E. coli* (DAEC)	Elaboration of α-haemolysin and cytotoxic necrotising factor 1 (CNF-1).	Watery diarrhoea	Supportive therapy

2.4.1.2. *Staphylococcus aureus*

S. aureus is a gram-positive coccus present in normal intestinal and skin flora of human and homoeothermic animal. Under define conditions, the pathogenic strains produces heat stable staphylococcal enterotoxins (SETs) and toxic shock syndrome toxins (TSST-1) (de Oliveira, 2010) both of which are known to induce diarrhoea.

Toxicity from SET results from the consumption of the preformed heat-stable enterotoxins (α-haemolysin) in contaminated food. Upon ingestion of the food contaminated with the SETs, the toxins results in signs of nausea, vomiting, fluid accumulation in ileal loops, and diarrhoea associated with fever (Rosengren *et al*, 2010; Perez-Bosque and Moreto, 2009). The main sources of *S. aureus* toxin contaminants are raw material and food processing unit such as human handling, water and environment (Linscott, 2011).. Serotonin receptor antagonists have been reported to ameliorate the vomiting, diarrhoea and prostration induced by SETs. The mechanism behind toxicity results from the activation of autonomous nervous system with resultant hyperperistalsis as well as activation of central pathways which control vomiting and diarrhoea (Podewils *et al*, 2004).

In contrast, TSST-1 is characterized by sudden onset of fever, vomiting, diarrhoea, erythematous rash with skin peels, hypotensive shock, impairment of renal and hepatic functions, and sometime death. Toxicity results via the production of pro-inflammatory cytokines and chemokines. Toxicity is usually exacerbated by further interaction between the activated immune system and inflammatory mediators (Krakauer *et al*., 2001).

2.4.1.3. *Campylobacter jejuni*

C. jejuni is an invasive Gram-negative, spiral-shaped rod bacterium present in the GIT of mammals, birds and primates (Lengsfeld *et al*., 2007). The major source of *Campylobacter* infection in mammals is from poultry and poultry products (Podewils *et al*, 2004). The clinical signs of *campylobacter* infections include pyrexia, abdominal pains, watery diarrhoea and dysentery (Podewils *et al*, 2004). The characteristic mechanisms *Campylobacter* infection involves invasion and translocation of the epithelium with a concomitant induction of inflammation (Hu *et al*., 2008).

2.4.1.4. *Shigella* spp

Shigella (*Shigella flexneri, Shigella dysenteriae, Shigella sonnei and Shigella boydii*) is a Gram-negative rod, non-motile and facultative anaerobic bacterium that invades the colon with resulting inflammation and diarrhoea (Podewils *et al*, 2004). *Shigella flexneri* is responsible for dysenteric symptoms and persistent illness while *Shigella dysenteriae* type-1 produces Shiga-toxin like EHEC causes bloody diarrhoea (Podewils *et al*, 2004). *Shigella sonnei* causes bacterial gastroenteritis and bacillary dysentery and *Shigella boydii* causes fever, chills, abdominal pain and diarrhoea.

2.4.1.5. *Vibrio cholerae*

V. cholerae is a motile, facultative anaerobic Gram-negative rod associated with potentially fatal diarrhoea (Granum, 2006) that results from the ingestion of the cholera enterotoxins (CT) from contaminated water and

seafood (Podewils *et al*, 2004). Watery, colourless mucous- flecked stool and vomiting are the main clinical signs associated with cholera which in severe cases can result in a life-threatening fluid and electrolyte imbalance (Podewils *et al*, 2004). Pathophysiologically, toxicity results from the CT induction of intestinal hypersecretion through the activation of the mucosal epithelium cAMP-adenylate cyclase system in the mucosal epithelium (Casburn-Jones and Farthing, 2004).

In addition, it has been speculated that ROS/RNS production in *V. cholerae* infection could also contribute to intestinal damage through lipid peroxidation of the cellular and mitochondrial membrane thereby further increasing membrane permeability and fluid loss (Gorowara *et al*., 1998). Other species of *Vibrio* such as V. *parahaemolyticus* and *V. vulnificus* also caused watery diarrhoea, abdominal cramps, nausea, vomiting. These organisms infect host from raw or undercooked seafood or cooked seafood contaminated with seawater (Linscott, 2011).

2.4.1.6. *Bacillus cereus*

B. cereus is a sporulating bacterium that causes both food poisoning and non-gastrointestinal infection (Al-khatib *et al*., 2007; Ramarao and Lereclus, 2006). In food poisoning, two main types of diseases namely diarrhoeal and emetic food poisoning are common. The diarrhoeal type of *B. cereus* food poisoning is caused by enterotoxins such as haemolysin BL (HBL), non-haemolytic enterotoxin (NHE) and cytotoxin K (CytK) (Lund *et al*., 2000)..with clinical signs of abdominal pain with diarrhea. Causes of the diarrhoeal forms always occurs from accidental contamination of food like meat, vegetables, pasta, deserts cakes, sauces and milk (Linscott, 2011).

In constrast the emetic form is induced by a small preformed heat and acid stable cyclic peptide (cereulide) (Agata *et al*., 1995; Ehling-Schulz *et al*., 2004) with clinical symptoms of sudden onset of nausea and vomiting, with or without diarrhoea (Linscott, 2011). The major sources include cooked foods, like meat or fried rice that have not been properly refrigerated. While the other species of *Bacillus* such as *B. subtilis*, *B. licheniformis*, *B pumilus* and *B. megaterium* are usually considered relatively safe, but they can also produce enterotoxins and emetic toxins involved in foodborne illness (From *et al*, 2007)

2.4.1.7. *Yersinia* species

Yersinia species are Gram-negative facultative anaerobic nonsporing rods or coccobacilli bacteria belonging to the Enterobacteriaceae family. Three human pathogenic species namely: *Y. pestis*, *Y. enterocolitica*, and *Y. pseudotuberculosis* are recognized (Fallman and Gustavsson, 2005). Y. *pestis* is the causative agent of bubonic plague characterized with the onset of fever, chills, headache, and weakness, followed by swelling and tenderness of lymph nodes while *Y. enterocolitica and* Y. *pseudotuberculosis* cause an enteric infection in humans called yersiniosis with clinical signs such as diarrhoea, vomiting, fever and abdominal pain (may mimic

appendicitis) following ingestion from undercooked pork, unpasteurized milk, tofu, contaminated water, chitterlings (Linscott, 2011, Damme *et al.*, 2010).

2.4.1.8. *Listeria monocytogenes*

L. monocytogenes is a Gram-positive bacterium which causes life-threatening invasive diseases referred to listeriosis in human and animals (Chaturongakul *et al.*, 2008; Todd and Notermans, 2011). Upon ingestion of the bacteria from contaminated foods such as unpasteurized milk, soft, cheese made with unpasteurized milk Linscott, 2011), the organism may, colonize the intestinal tract with resultant diarrhoea (Chaturongakul *et al.*, 2008).

2.4.1.9. *Clostridium* spp

C. difficile is an anaerobic, spore-forming, Gram-positive bacillus widely distributed in the environment and present in the colon flora of less than 3% of healthy adults (Beaugerie *et al.*, 2003). *C. difficile* causes a spectrum of diseases ranging from benign diarrhoea to fatal colitis and most often as a consequent of antibiotics treatment. Most antibiotics predispose *C. difficile* overgrowth leading to the production and accumulation of and diarrhoea are Toxins A (enterotoxin) and B (cytotoxin) in the intestine. Both toxins A and B inactivate intracellular Rho-proteins by glycosylation, leading to desorption of the cytoskeleton, production of inflammatory cytokines and damage to tight junctions The most commonly antibiotics associated with *C. difficile* overgrowth include cephalosporins, clindamycin and broad-spectrum penicillins (Wistrom *et al.*, 2001).

In contrast, *C. perfringens* is an important food poisoning bacterium with clinical sign as diarrhoea, abdominal cramping and nausea. The main sources of infection include contaminated meat, poultry, gravy and inadequately reheated food (Linscott, 2011). *C. botulinum* may also play a role in diarrhoeal diseases when the preformed botulinum toxin is consumed from improperly canned foods, herb-infused oils, baked and potatoes in aluminium foil. Symptoms of infection include abdominal cramping, nausea, vomiting, diarrhea, double vision, long term nerve damage and possible even death from paralysis (Linscott, 2011).

2.4.1.10. *Salmonella typhimurium*

S. typhimurium is a bacterium that may be associated with mild gastroenteritis to enteric (typhoid) fever, bacteraemia and septicaemia commonly referred to as salmonellosis (Mastroeni and Maskell, 2006). The clinical signs of salmonellosis include diarrhoea, fever and abdominal cramps. In people with typhoid fever, *Salmonella* spreads systemically from the gut to blood stream and other parts of the body resulting in mortality if not treated adequately with antibiotics (Castillo *et al.*, 2011). The virulence of the *Salmonella* bacterium differs among the different animal species depending on *Salmonella* serovar involved, strain, infective dose, host animal species,

age and immune status of the host (Castillo *et al.*, 2011). The pathogenesis *of Salmonella* involves adhesion/invasion to specific intestinal epithelial cells, mainly in the ileum (Guttman and Finlay, 2009).

2.4.1.11. *Enterococcus faecalis*

E. faecalis is a gram-positive bacterium that survives symbiotically in the human or animal's intestinal tract. However, under conditions such as the disruption delicate host-commensal relationship following antibiotic use, abdominal surgery or changes in host immunity, the enterococci becomes invaders of the intestinal wall (Butler, 2006) through the production of adhesin, aggregating and binding substances (Butler, 2006). *E. faecalis* is known to produce superoxide (O_2^-) that can results in hydroxyl radical formation which contributes to oxidative stress in the intestine and membrane lipid peroxidation (Huycke and Moore, 2002; Sun *et al*, 2010; Peluso *et al.*, 2002; Granot and Kohen, 2003) (Fig 2.5).

2.5. Fungal induced diarrhoea symptoms

2.5.1. *Candida albicans*

C. albicans is a yeast fungus and exist as a member of normal flora in the GIT and mucocutaneous membrane (Forbes *et al.*, 2001). However, following the use of antibiotic therapy that result in sterilization of the GIT flora, *C. albicans* can overgrowth to take the place of removed organisms with end result of diarrhoeal symptoms(Henry-Stanley *et al.*, 2003). Other predisposing factors include altered intestinal permeability and diminished host immunity response. It has been postulated that this organism produces virulence factors which increases fungal adherence to host cells, fungal secretion of proteolytic enzymes and fungal morphological switching (ability to change and grow in several distinct morphological forms: yeast, hyphae, and pseudohyphae, according to environmental conditions) (Henry-Stanley *et al.*, 2003). Clinical signs associated with enteric candidiasis are abdominal pain, cramping, rectal irritation and absence of nausea, vomiting, bloody and mucus stool, and pyrexia (Levine *et al.*, 1995).

2.6. Viral induced diarrhoea

2.6.1. Rotavirus

Rotavirus is a major cause of severe diarrhoea and account for 30% and 80% of all cases of acute gastroenteritis (Savi *et al*, 2010). The diarrhoeal mechanism of the organism includes the production of enterotoxin NSP4 which induced Na^+-glucose dependent malabsorption and destruction of enterocytes (cytotoxicity). The toxin also has a direct effect on the intestinal barriers by blocking TJs formation with resultant diarrhoea through a 'leak flux' mechanism in which water is secreted into the lumen of the intestine (Dickman *et al.*, 2000).

2.6.2. Norovirus

Norovirus is considered one of the major global causes of gastroenteritis (Mattison, 2010) The diseases is opportunistic and is usually transmitted through faecal contamination of food, water or contact with an infected host following poor hygiene. The organism has the ability to infect small intestine and induce intestinal TJ dysfunction, malabsorption through villus surface area reduction and an increased number of cytotoxic intraepithelial lymphocytes (Troeger *et al.*, 2009). Clinical signs associated with infection are nausea, vomiting, diarrhoea and abdominal pains (Koopmans, 2008).

2.6.3. Hepatitis A virus

Hepatitis A is a small, non-enveloped spherical with cubic symmetry, thermostable and acid resistant virus. While the primary target organ of infection is the liver, the resultant hepatitis (Koff, 1998) result in clinical signs dark urine, jaundice, malaise, weakness, fever, anorexia, nausea and vomiting, abdominal pains, and diarrhoea (Koff, 1992). Sources of infection usually result from contaminated water on raw produce, food, and shellfish or exposure to the water itself (Linscott, 2011).

2.6.4. Human immunodeficiency virus (HIV)

Chronic diarrhoea is one of the complications associated with HIV infection and acquired immune deficiency syndrome (AIDS) due to multiple enteric opportunistic microbes (DuPont and Marshall, 1995). While HIV is important in secondary enteric diseases as a result of immune suppression (CD4+ T-lymphocytes destruction), the virus can result in diarrhoea directly by altering the mucosa structural arrangement viz referred to as HIV-enteropathy (Epple *et al.*, 2009). The diarrhoea resulting from HIV appears to be caused by the releases of cytokines from the infected immune cells (Schmitz *et al.*, 2002).

2.7. Protozoa induced diarrhoea

2.7.1. *Giardia intestinalis*

G. intestinalis (syn. *Giardia doudenalis, Giardia lamblia)* is a flagellate protozoa parasite of the upper small intestine that exists as a motile trophozoite (Cotton *et al.*, 2011). The organism colonizes the small intestinal lumen and induces non-inflammatory and malabsorptive diarrhoea (Schulzke *et al.*, 2009). The pathophysiology of Giardiasis involves Na+-dependent D-glucose absorption impairment, active electrogenic anion secretion activation, mucosal inflammation and leak flux (Buret, 2007; Troeger *et al.*, 2007). Clinical signs of *Giardia* infection include bloating, steatorrhea and nausea. Chronic infection cause weight loss, growth retardation and development in young children

2.7.2. *Entamoeba histolytica*

E. histolytica is a protozoa parasite which infects the large intestine with resultant intestinal dysfunction characterized by invasive illness and severe dehydration commonly referred to as amoebiasis (Ralston and Petri, 2011). The pathophysiology of amoebiasis involves villus structural destruction, increased epithelial permeability and alteration of TJs (Lauwaet *et al.*, 2004). The organism also causes invasive disease such as colitis and abscess through massive host tissue destruction. The clinical signs are usually similar to *S.dysenteriae* or enteroinvasive *E. coli* with blood and pus contaminated stool. Other related infectious species include *E. dispar* and *E. moshkovskii* (Ralston and Petri, 2011).

2.7.3. *Cryptosporidium parvum*

C. parvum is an intracellular protozoa parasite that infects epithelia causing cryptosporidiosis (Kenny and Kelly, 2009) which manifest clinically as profuse watery diarrhea, containing mucus, but rarely blood or leukocytes (O'Hara and Chen, 2011). Some other clinical signs of cryptosporidiosis include nausea, vomiting, cramp-like abdominal pain and mild fever (Linscott, 2011). The period and severity of clinical symptoms of intestinal cryptosporidiosis depends on the immune status of the infected individual (Linscott, 2011). Cryptosporidiosis in the healthy individuals is usually a self-limiting illness with approximate duration of 9-15 days while in immunocompromised patient the infection in severe and life-threatening (O'Hara and Chen, 2011).

2.7.4. *Cyclospora cayetanensis*

C. cayetanensis is a protozoan parasite which invades the epithelial cells of the small intestine upon ingestion of sporulated oocysts in contaminated food or water (Chacin-Bonilla, 2010; Manfield and Gajadhar, 2004). Pathomechanisms of *C. cayetanensis* infection are intestinal inflammation associated with pathological lesions such as villus blunting, and malabsorption. The clinical signs of the infection include watery diarrhoea, loss of appetite, weight loss, abdominal bloating and cramping, increased flatulence, nausea, fatigue, and low-grade fever (Linscott, 2011).

2.8. Parasitic induced diarrhoea

2.8.1. *Trichinella spiralis*

T. spiralis is a food-borne zoonotic parasite responsible an enteral phase and a muscular phase (Cui *et al.*, 2011). The adult worms live in the duodenal and jejunal mucosa of flesh-eating animals and humans, while the larvae live in skeletal muscles of the same hosts (Cui *et al.*, 2011). The source of infection is raw or undercooked contaminated meat (pork, bear, walrus and moose), cross-contaminated ground beef and lamb (Linscott, 2011). The clinical intestinal symptoms manifest one or 2 days after ingesting the contaminated meat due to the matured adults penetrating the intestinal mucosa, resulting in nausea, abdominal pain, vomiting, and diarrhoea

(Linscott, 2011). *T. Spiralis* induced changes in intestinal function by hypersensitivity mechanism resulting in an increased intestinal chloride and fluid secretion (Cui *et al.*, 2011).

2.9. Immune disordered induced diarrhoea

2.9.1. Compromised immune system

The main function of the immune system is to protect against disease through the recognition and removal of pathogens or their sequelae (Gertsch *et al.*, 2011). To fulfil this role, the body make use of an innate immune system that defends it in a non-specific manner via molecular interaction and inflammation (Gertsch *et al.*, 2011) and an adaptive system comprised of specialized effector cells (T and B cells) which not only recognize antigens but play a role in their active removal (Gertsch *et al.*, 2011). In the GIT, the important protective system which prevent the normal flora from becoming pathogenic are gut-associated lymphoids tissue (GALT), epithelial-derived antimicrobial peptides (AMPs) (such as defensins, cathelicidinand lysozyme present in the secretion which constantly washes the mucosal surfaces), the mesenteric lymph nodes, the liver's Kupfer cells, mast cells, within the intestinal walls and the reticuloendothelial system of the intestinal walls. In diseases characterized by immune suppression such as HIV, the immune system is destroyed which makes a person more susceptible to infectious diseases (Gertsch *et al.*, 2011). One of the clinical signs that may result is diarrhoea as the above-mentioned micro-organisms colonize the compromised GIT.

2.9.2. Hyperactive immune system

The normal response of the immune system during conditions of antigen stimulation is generally an inflammatory response with the release of numerous inflammatory mediators (interferon-γ, tumour necrosis factors (TNF-α), interleukin-6 (IL-6), and IL-1β) which in conjunction plays a role in the removal of the causative agents (Sprague and Khalil, 2009). On the removal of inciting cause, the inflammatory response is usually terminated. However, on occasion when the body failed to terminate the inflammatory response, the inflammatory agents can be equally as destructive to the host's own tissues. The latter usually result from the damage of the epithelial mucosa cells, from the generated ROS/RNS and subsequent lipid peroxidation. With the destruction of the epithelial cells, the body loses absorptive capacity with resultant diarrhoea. The produced cytokines also has the ability to directly increase intestinal mucosa permeability and fluid loss. Inflammatory bowel disease is one of the diseases caused by up-regulated immune system (Gertsch *et al.*, 2010).

2.10. Antibiotic therapy induced diarrhoea

Diarrhoea develops in some patient following antibiotic chemotherapy with one of the following mechanisms:

- Antibiotic toxicity: Some antibiotic (levofloxacin, azithromycin and amoxicillin-clavulanate) may have a direct negative effect on the GIT with resultant poor absorption or enteropathy characterized by

infiltration of the lamina propria by macrophage (Dobbins, 1968, Hartmut, 2010). In addition, antibiotic such as erythromycin has prokinetic action on the GIT, mediated through motilin receptor stimulating potential (Catnach and Fairclough, 1992, Annese *et al.*, 1992).

- Alteration of digestive function: The removal of some commensal organisms by antibiotic (Hofmann, 1977) could result in decreased carbohydrate digestion (Saunders and Wiggins, 1981) which leads to accumulation of these osmotically active substances in the intestinal lumen. The net result is the accumulation of electrolytes and water from osmotic pull in the intestinal lumen which is evidence as diarrhoea (Beaugerie and Pettit, 2004).

- Overgrowth of pathogenic microorganisms: The use of antibiotic may result in the removal of beneficial GIT flora. As a result of the disruption of this balance ecosystem, various pathogenic organisms (as listed above) can overgrow and colonise the GIT (Beaugerie and Pettit, 2004). The predisposing antibiotic for various diarrhoeal pathogens include cephalosporins, clindamycin and broad-spectrum penicillins for *C. difficile* (Wistrom, 2001; Stoddart and Wilcox, 2002); β-lactams or pristinamycin for *Klebsiella oxytoca* (Wu *et al.*, 1999) or tetracycline for *S. aureus*. *Salmonella* and *Candida* overgrowth can be a consequence of wide range of antibiotic with broad base actions (Danna, 1991; Gupta and Ehrinpreis, 1990).

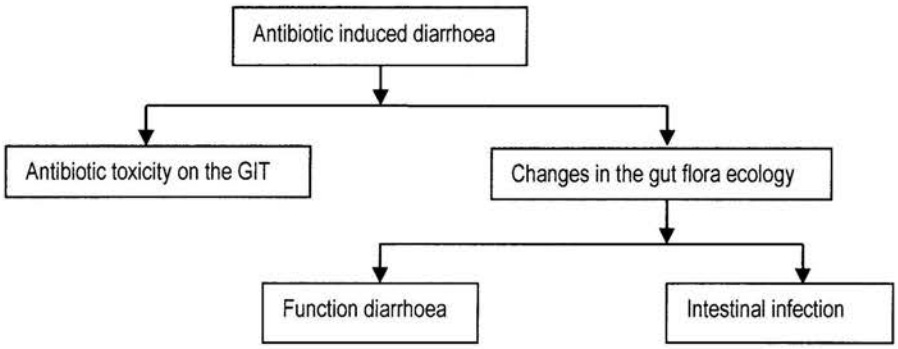


Fig. 2.8. Mechanism of antibiotic induced diarrhoea

2.11. Diabetic complications induced diarrhoea

Gastrointestinal disorders manifesting as diarrhoea (watery stool) or constipation (dry and hard stool) is a common symptom in the diabetic patient (Gould and Sellin, 2009) with a prevalence of approximately 12.5 to 32.4% and 60% respectively. In addition the oral hypoglycaemia medications used for the management of diabetes viz metformin, acarbose, miglitol (Forgacs and Patel, 2011),, exenatide and orlistat (Gould and Sellin, 2009) may also induced diarrhoea as side effect while the recommended dietary material such as the non-digestible sweeteners (sorbitol, mannitol and D-xylose) induce an osmotic diarrhoea Forgacs and Patel, 2011).

Bacterial overgrowth may also result in diabetic patient from decreased functioning of the immune system as described above (Forgacs and Patel, 2011).

2.12. Food allergy induced diarrhoea

Diarrhoea is one of the clinical manifestations of food allergy (Wang *et al.*, 2010). The mechanisms of action include active ion secretion, altered epithelial barrier function (Groschwitz and Hogan, 2009), and mucosal damage resulting in malabsorption and osmotic diarrhoea (Wang *et al.*, 2010). The initiation of food induced intestinal allergy is regulated by numerous inflammatory cells and mediators, including mast cells and T_H2 cytokines (IL-4, IL-5, and IL-13) (Wang *et al.*, 2010). The release of neurotransmitters (serotonin, histamine) and inflammatory mediator (COX-2 and LOX) by mast cell induced ion secretion in the presence of allergen (Schenk and Mueller, 2008). These neurotransmitters and inflammatory mediators also stimulate intestinal contractions (altered intestinal motility) which act synergistically with ion secretion to cause diarrhoea.

2.13. Potential mechanisms in the control of diarrhoea

2.13.1. Oxidative damage and antioxidants in diarrhoeal management

Several endogenous strategies are available in human and animal body to combat oxidative damage. These provide ways for normal oxidative metabolism to occur in the body without damaging the cells and also allow for normal ROS/RNS-mediated cellular response such as phagocytosis and intracellular signalling (Valko *et al*, 2007). Therefore, the possibility exists that returning the animal to a more neutral oxidative balance, may promote repair of damaged membranes (Nose, 2000). As a result antioxidants and/or radical scavengers may be beneficial in the attenuation of diarrhoea. The best known antioxidants as treatment are selenium, vitamin E, vitamin C and the proanthocyanidins in red wine and resverastrol in commercial grape seed extract.

2.13.2. Inflammation and anti-inflammatory agents in diarrhoea management

As a result of the negative impact the inflammatory cascade can have on the functionality of the GIT, modulation of these processes through the use of drugs may be of benefit. Possible mechanisms include attenuation of inflammatory process through the use of anti-inflammatory, antioxidative and radical scavenging mechanisms. Potential target include drugs with cyclooxygenase and lipoxygenase enzyme inhibitory activity. The drugs that are used commonly for this are non- steroidal anti-inflammatory drugs (NSAIDs) like indomethacin, aspirin, ibuprofen, diclofenac and coxibs.

2.13.3. Enteric nervous system in diarrhoea symptoms and treatment

The ENS is an important target for pharmacological intervention in diarrhoea through the use of agonists and antagonists that target these ENS endogenous receptors. Numerous pharmaceutical agents are currently

available for alleviating many of the clinical signs of diarrhoea. The effects and possible sites of pharmacological intervention against the activities of neurotransmitters in diarrhoeal symptoms are presented in Table 2.2.

Table 2.2: Neurotransmitters and modulators of ENS causing intestinal secretion in diarrhoea

Neurotransmitters	Effects on GIT	Receptors	Potential pharmacological intervention	modulator
Acetylcholine	Main endogenous excitatory neurotransmitter in the GIT.	Nicotinic and muscarinic receptor subtypes M_1 and M_3.	Non selective nicotinic acetylcholine receptor antagonist or specific muscarinic acetylcholine receptor antagonist	atropine
Serotonin	Modulate muscular contraction and relaxation, intestinal fliud secretion and enhanced colonic transit.	5-HT_3, 5-HT_4	5-HT_3 receptor antagonist (diarrhoea) and 5-HT_4 receptor agonist (constipation)	Metoclopramide, granisetron, ondansetro, tropisetron, palonsetron, alosetron, cisapride
Substance P	Transmitter of enteric neurones and extrinsic afferent fibre, control of GI motility, secretion, vascular permeability, immune function and pain sensitivity	NK_1, NK_2 and NK_3	NK_1 and NK_2 receptor antagonist	
Histamine	Modulation of GIT motility, enhancement of gastric acid secretion, increases mucosal Cl^- ion secretion, and modulator of immune functions.	H_1, H_2, H_3	H_1, H_2 antagonist	Cimetidine and ranitidine
Opioid peptide	decrease motility, increase transit time, increase fluid absorption from the intestine	Mu (μ),delta (δ) and kappa (κ)	μ, δ agonist	loperamide racecadotril, diphenoxylate
Nitric oxide	ENS neurotransmitter, pro-absorptive and secretory agent		iNOS inhibitor, NO modulator	N^G-nitro-l-arginine methyl ester (L-NAME
Dopamine	Prokinetic and antiemetic, enhance antral contractility and inhibit fundus receptive relaxation	D_2	antagonist	Domperidone
Motilin	induced antral contractility			Erythromycin, motilides, motilactides

2.14. Plants as potential source of therapeutic agents in alleviating diarrhoeal symptoms

Due to the widespread occurrence of diarrhoea as a disease together with the prevalence coinciding with human development, plants and fungi have featured widely in the management of the disease. Their use has become so common in human and veterinary medicines that a number of compounds considered to be allopathic are of natural origin. A non-exhaustive list includes:

- Antioxidant: The natural vitamins and red pigments present in plant.
- Anti-inflammatory: Salicylic acid from willow bark.
- ENS modifiers: Atropine from *Atropa belladonna*, tincture of opium from *Papaver somniferum*.
- Antibiotic: By definition all antibiotics are natural product produced by fungi. Almost all the available classes of antimicrobials are of fungal origin.

Alongside these naturally derived allopathic medicines, medicinal plant have been widely use in alleviating diarrhoeal symptoms in humans and animals (Brijesh *et al.*, 2006; Gutierrez *et al.*, 2007). Numerous species of these plants have been screened and validated for their use in treatment of diarrhoea (Gutierrez *et al.*, 2007).

2.14.1. Anti-infectious mechanisms of plant secondary metabolites against diarrhoeal pathogens

- Antimicrobial: Many plant metabolites are known to exhibit some level of toxicity toward microorganisms. Numerous mechanisms of actions have been hypothesized to explain their antimicrobial activity such as microbial enzymes inhibition, deprivation of essential growth substrates, cell membrane disruption (Cowan, 1999) or direct interference with metabolic pathways.
- Antiadhesion: Adhesion of some enteric pathogen to the mucosa epithelium of the host cells is the first important step in intestinal infections that may lead to the development of diseases (Ofek and Sharon, 1990). Application of antiadhesives chemotherapy can be effective only against microorganisms that depend on the surface contact with host cells as prerequisite for survival, multiplication and virulence (Lengsfeld *et al.*, 2007).
- Antitoxin: Since enteric pathogens may induce diarrhoea through the production of toxin (endotoxin or cytotoxin) the neutralization with plant antidiarrhoeal compounds may beneficial in the management of diarrhoea. Activated charcoals processed from plants are also used as toxin binders. Other binder includes pectin obtained from apples.
- Immunomodulatory: With immune suppression being a pre-disposing, drugs or medicinal plant preparations with immune stimulating activities may help in attenuating many infectious diseases.

2.14.2. Antioxidative mechanisms of plant phytochemical as potential antidiarrhoeal agents:

- Free radical scavenging: Many plant preparations and phytochemicals have strong antiradical activities which can antagonize the deleterious action of free radical. The mechanism may be electron transfer or hydrogen donating to stabilized the free radical molecules generating radicals that are relatively stable due to delocalization resulting from resonance and unavailability of site for further attack by molecular oxygen (Mello *et al*, 2005).
- Complexation of catalytic metallic ion: Metallic ions such as ferrous ion (Fe^{2+}), cuprous ion (Cu^{2+}), Manganese ion (Mg^{2+}) and Zinc ion (Zn^{2+}) can also generate free radicals (Kane, 1996). Many plant molecules moderate oxidation activity by complexing with the free transition metal thereby inactive their capacity to catalyse oxidative process.
- Pro-oxidation enzymatic pathways: With the generation of oxidant being enzyme driven, the antioxidant activities of plant phytochemical may be able to inhibit these enzymatic pathway or inactivation of the enzyme.
- Lipid peroxidation inhibition: Scavenging of free radicals is one of the major antioxidation mechanisms to inhibit the chain reaction of lipid peroxidation and reduction of the deleterious effect of the cytotoxic products.
- Inhibition of nitric oxide (NO): NO generated by inducible NOS (iNOS) can act synergistically with other inflammatory mediators in the development of diarrhoea. The inhibition or down-regulation of iNOS expression may be beneficial to reduce the inflammatory response.

- Immune system optimization: Over expression of immune system may cause damage to surrounding tissues and consequently results in a host of diseases and illness including diarrhoea. Many medicinal plants and phytochemical compounds protect against oxidative stress due to immunomodulatory activity (Wang *et al*., 2002).

2.14.3. Anti-inflammatory mechanisms of plant phytochemical in diarrhoea management:

- Cyclooxygenase inhibition: Compounds with COX enzyme inhibitory potential could be use as anti-inflammatory agents. Some plant secondary metabolites such as alkaloids, phenols, , terpenoids and their derivatives have potential to inhibit the formation of pro-inflammatory signalling molecules such as prostaglandin (Polya, 2003)
- Lipoxygenase inhibition: Lipoxygenase metabolites are critical mediators of inflammation and thus important in the pathogenesis of abdominal distress and diarrhoea associated with intestinal inflammation. Plant phytochemicals with lipoxygenase inhibitory potential are candidate for anti-inflammation and the resulting diarrhoea.

- Modulation of cytokines activity: A number of diarrhoea pathogenesis causes severe intestinal inflammatory with hypersecretion of pro-inflammatory cytokines (MacNaughton, 2006). Inhibition of the pro-inflammatory cytokine mediators can remove their negative activities associated with gastrointestinal disorders including diarrhoea.

2.14.4. Antidiarrhoeal mechanisms of plant phytochemicals

- ✓ Antispasmodic: Spasmolytic agents are used in the treatment hypermotility of the GIT while prokinetic agents are used in treatment of hypomotility (Gilani, 2005). Many phytochemicals demonstrated various range of spasmolytic or antispasmodic activities against spontaneous or agonist induced contraction on isolated parts of the GIT.
- ✓ Antisecretory: Microbial enterotoxins cause diarrhoea by disturbing the balance between intestinal absorption and secretion in favour of the later. Therefore, inhibition of the intestinal secretion is one therapeutic model for treating diarrhoea (Velazquez *et al*., 2009).

2.15. Classification of phytochemicals with antidiarrhoea potential

2.15.1. Terpenoids

Terpenoids are the most structurally diverse groups of natural products formed by fusion of isoprene monomers in plants. This class of plant secondary metabolites are grouped according to the number isoprene units or numbers of carbon in their skeletal structure (Zwenger and Basu, 2008). The group include monoterpenes which contain two units of isoprene with C_{10} and are present as essential or volatile components of herbs, spices and flowers. Sesquiterpenes are derivatives of three isoprene units containing 15 carbon atoms in their structures and are present in essential oil. This group of compounds acts as phytoalexins, antimicrobial and antifeedant in plants. Diterpenes contains 20 carbon atoms derived from four units of isoprene and pharmacological activities such as taxol (anticancer) and forskolin (for treating glaucoma). Triterpenes are contains 30 carbon atoms skeleton formed by the head-to-head joining of two C_{15} chains, each of which contains three isoprene units joined head-to-tail. Tetraterpenes such as carotenoids are 40 carbon atoms made of 8 isoprene units.

Several terpenoids have been identified so far to have good activity in one or more of the antidiarrhoeal mechanisms described above. Some of the compounds include:

- Niloticane from *Acacia nilotica* (L.) Willd ex Del. subsp. *kraussiana* (Benth) Brenan (Fabaceae) had inhibitory effect on (*Bacillus subtillis*, *Staphylococcus aureus*, and *Escherichia coli* at 4.0, 8.0 and 33.0 µg/ml respectively) (Eldeen *et al*, 2010).
- 1,3-Dihydroxy-12-oleanen-29-oic acid; 3,30-Dihydroxyl-12-oleanen-22-one; 1,3,24-Trihydroxyl-12-olean-29-oic acid; 1,23-Dihydroxy-12-oleanen-29-oic acid-3-*O*-2,4-di-acetyl-1-rhamnopyranoside

isolated from *Combretum imberbe* have been reported to have MIC of 16.0 µg/ml against *E. coli* (Angeh *et al.*, 2007).

- Oleanolic acid, ursolic acid, and betulinic acid from *Chaenomeles speciosa* have the potential of blocking the binding of virulence heat labile unit B (LTuB) of *E. coli* enterotoxin to ganglioside receptor (GM 1). The IC_{50} values enterotoxin binding activity were 202.8±47.8, 493.6±100.0, and 480.5±56.9 µM respectively (Chen *et al.*, 2007).
- Glycyrrhizin from *Glycyrrhiza uralensis* have been reported to have LTuB-binding inhibitory activity with IC_{50} of 3.26±0.17 mM, therefore, can suppress LT-induced intestinal fluid accumulation (Chen *et al.*, 2009).
- Oleanolic acid and echinocystic acid both isolated from *Luffa cylindrica* (cucurbitaceae) were reported to increase phagocyte index, stimulate macrophage, increase humoral and cell-mediated immune responses (Khajuria *et al.*, 2007).

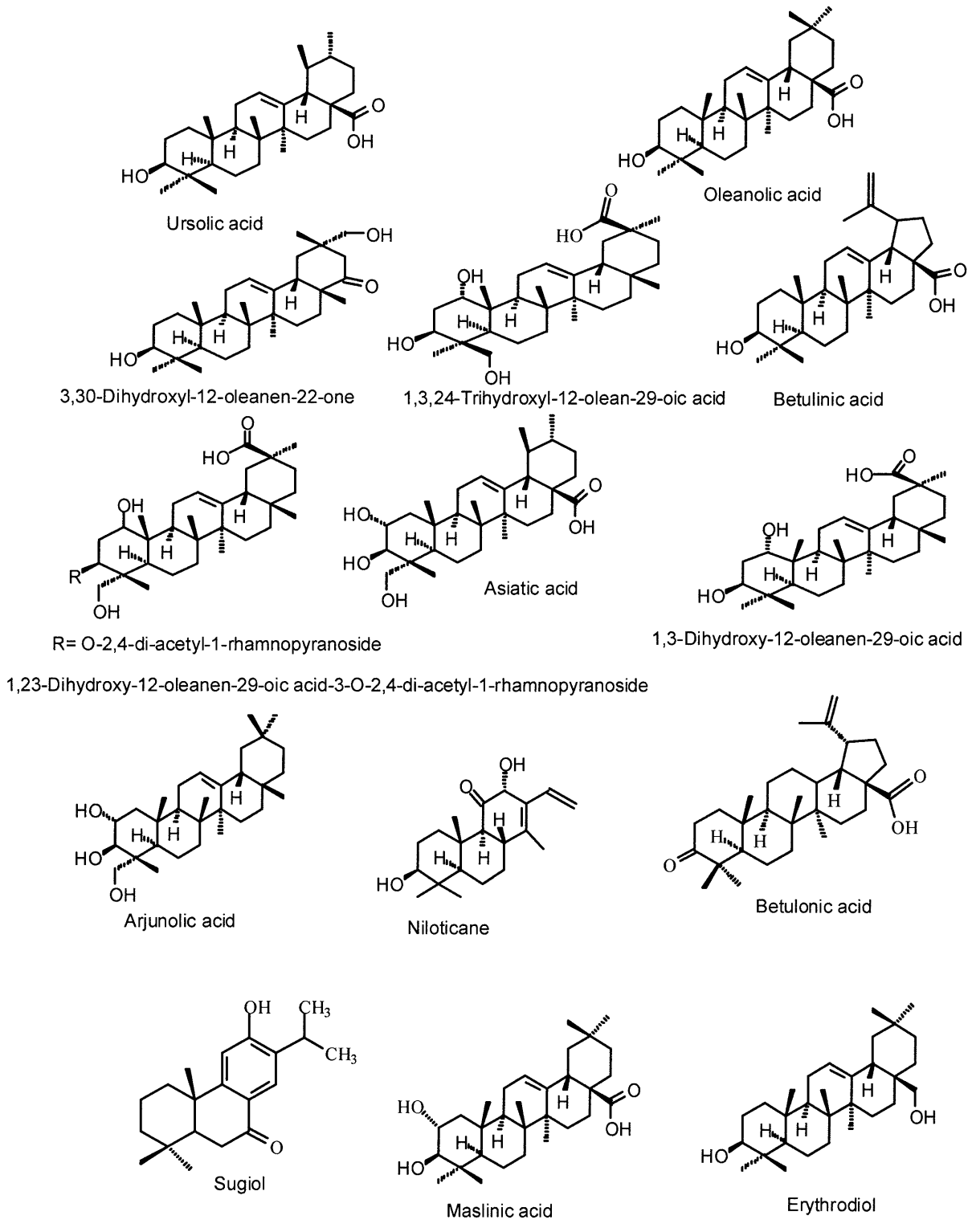


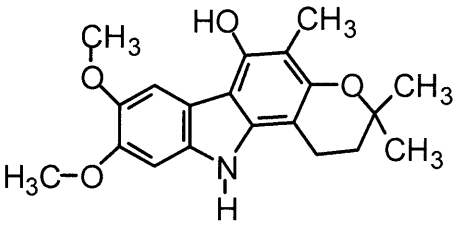
Fig. 2.9. Chemical structures of bioactive terpenoids against diarrhoeal mechanisms

- Sugiol (a diterpenoid) isolated from *Amentotaxus formosana* reported to exhibit good xanthine oxidase inhibitory activity with IC_{50} of 6.8±0.4 µM compared to standard allopurinol with IC_{50} of 2.0±0.7 µM (Lin *et al*., 2010).
- *Epi*betulinic acid and betulonic acid isolated from *Maytenus cuzcoina* Loesener demonstrated nitric oxide inhibition of 89.1±4.4% (IC_{50} of 0.7 µM) and 69.2±5.1% (IC_{50} of 0.3 µM) respectively in vitro (Reyes *et al*., 2006).
- Betulonic acid isolated *Maytenus cuzcoina* Loesener to have demonstrated PGE inhibition activity of 58.4±3.9% (IC_{50} of 2.7 µM) (Reyes *et al*., 2006) in vitro.
- Maslinic acid, oleanolic acid, erythrodiol and uvaol isolated from olive pomace oil was shown to have concentration dependent IL-6, TNF-α modulatory effects in a human mononuclear cells culture assay (Marquez-Martin *et al*., 2006).
- Ganoderic acids C and D isolated from *Ganoderma lucidum* have been reported to have anti-allergic properties through histamine release inhibitory mechanisms (Rios, 2008). Friedoolean-type triterpenoid (bryonolic acid) (Rios, 2005) and cucurbitacin structure, including dihydrocucurbitacin B (Escandell *et al*., 2007) and cucurbitacin R (Escandell *et al*., 2010) from *Cayaponia tayuya* (Cucurbitaceae) were reported to exhibit anti-allergic activities through inflammatory responses modulation.

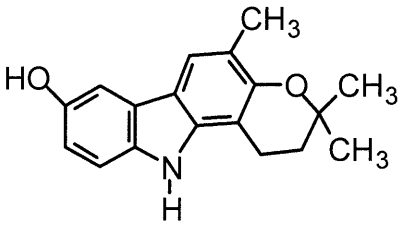
2.15.2. Alkaloids

Alkaloid refers to a group of heterocyclic nitrogen compounds and many exhibit remarkable physiological and pharmacological activities (Samy and Gopalakrishnakone, 2008). Most alkaloids are derived from amino acid precursor and are classified based on their structure as pyridine, tropane or pyrrolizidine alkaloids. Though, alkaloids have many pharmacological mechanisms such as microbiocidal effect on diarrhoeagenic pathogens, the main antidiarrhoea effect is probably that of delayed intestinal transition of bowel materials (Cowan, 1999).
Some of the pharmacological important antidiarrhoeal alkaloids include:

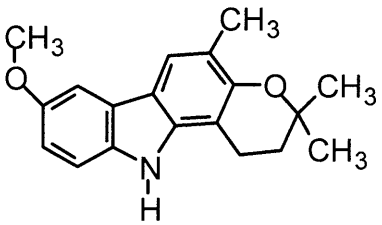
- Kurryam, koenimbine and koenine isolated from *Murraya koenigii* Spreng (Rutaceae) seed were reported to be active against various diarrhoeal mechanisms such as castor-induced diarrhoea, GIT motility, PGE_2 induced enterpooling (Mandal *et al*., 2010).
- 8-acetonyldihydronitidine and 8-acetonyldihydroavicine both isolated from *Zanthoxylum tetraspermum* had strong antistaphyloccocal activities with MICs of 1.56 and 3.12 µg/ml respectively. However, the two compounds were reported to have no inhibitory activity against *E. coli* (Nissanka *et al*., 2001).
- Boldine isolated from *Peumus boldus* Molina and *Corydalis ternate* Nakai had good antioxidant properties, indicating the effectiveness of the compound in preventing various oxidative-stress related illnesses like inflammatory cascades, immune dysfunctions. However, at high concentration, it causes cellular damage and potentiates lipoperoxidation, which is a pro-oxidant property.



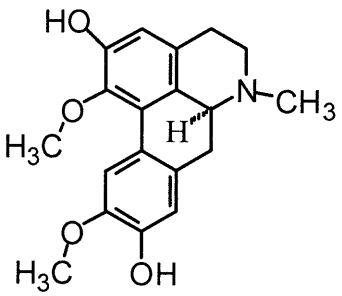
Kurryam



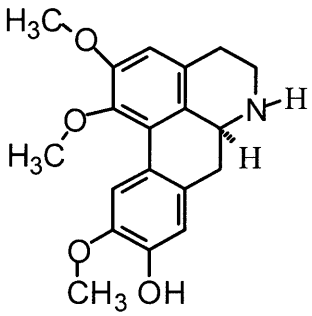
Koenimbine



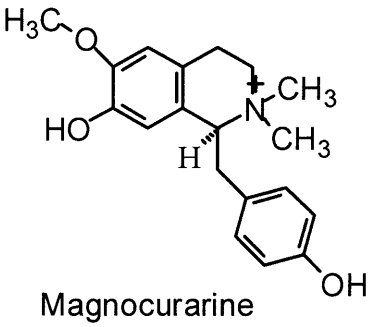
Koenine



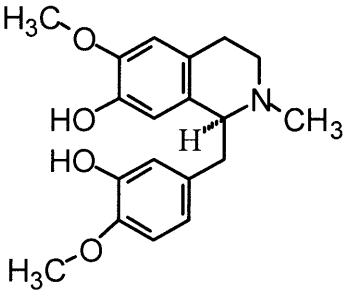
(S)-boldine



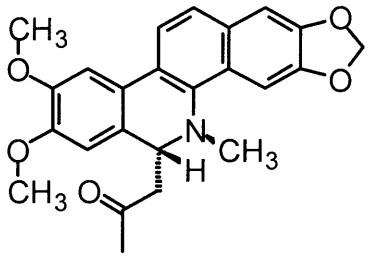
Laurotetanine



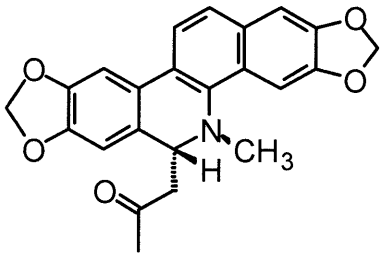
Magnocurarine



(S)- reticuline



8-acetonyldihydronitidine



8-acetonyldihydroavicine

Fig. 2.10. Chemical structures of bioactive alkaloids against diarrhoeal mechanisms

2.15.3. Phenolic

Phytophenolic compounds are widely distributed as secondary metabolites of medicinal plants, as well as in some edible plants (Naczk and Shahidi, 2004). The consumption of diet rich in phenolic compounds has been hypothesized to be important in health promotion and disease prevention in humans and animals (Ramful *et al*, 2010). Phenolic compounds are characterized as aromatic metabolites that have one or more acidic hydroxyl groups attached to the phenyl ring. The sub-class of phenolics compounds are presented in Fig. 2.8. This group of compounds exhibits numerous biological activities directly or indirectly on intestinal epithelium which contribute to alleviation of diarrhoea symptoms.

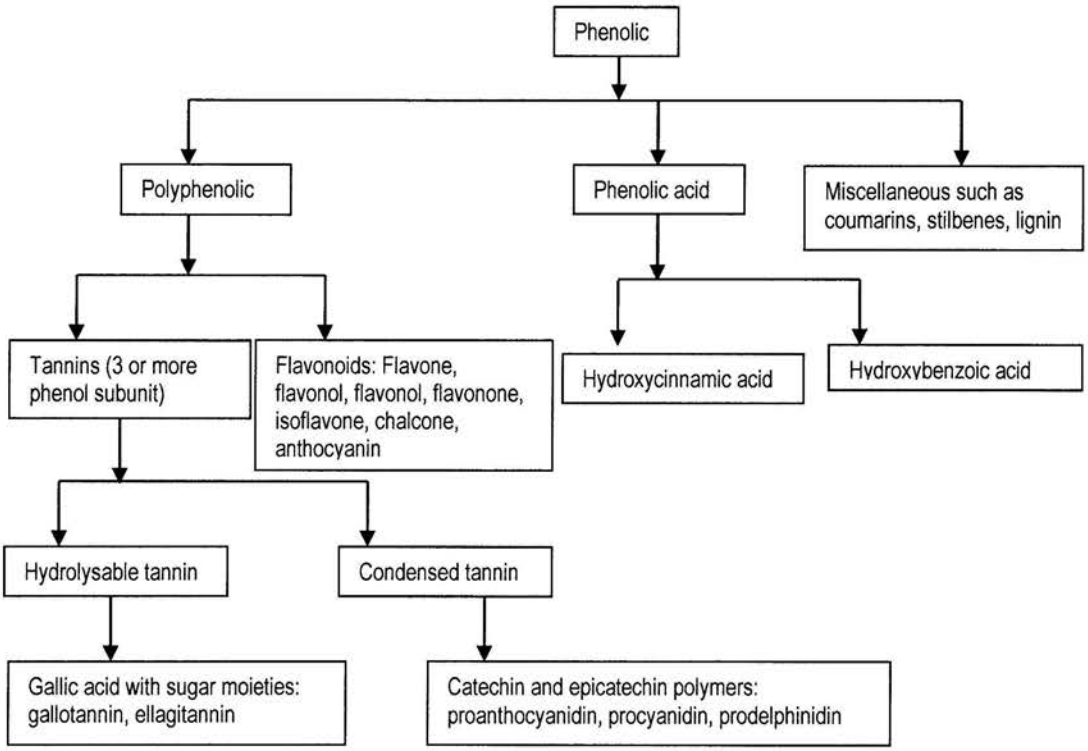
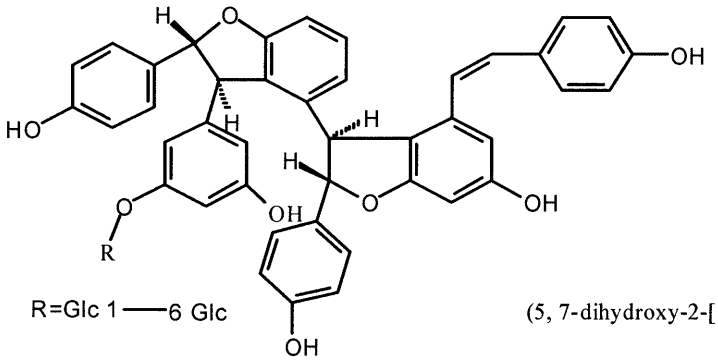


Fig. 2.11 Sub-classes of biologically important group of phenolic compounds.

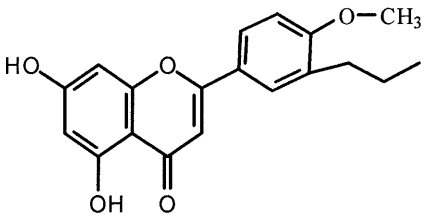
Phenolic compounds with antidiarrhoeal activities against some of the mechanisms described above include:

- 2(*S*) -5'- (-1'",1"'-dimethylallyl) -8- (3",3"-dimethylallyl) -2',4',5,7-tetrahydroxyflavanone; 2(*S*) -5'- (1"',1"'-dimethylallyl) -8- (3",3"-dimethylallyl) -2'-methoxy-4',5,7-trihydroxyflavanone and 5'- (1"',1"'-dimethylallyl) -8- (3",3"-dimethylallyl) -2',4',5,7-tetrahydroxyflavone] from *Dalea scandens* var. *paucifolia* with MIC of 1.56 µg/ml against standard and Methicillin-resistant *S. aureus* (MRSA) (Nanayakkara *et al.*, 2002).
- Moracin T isolated from *Morus mesozygia* was reported to exhibit antimicrobial activities with MIC of 5.0 µg/ml against *E. coli*, S. *dysenterica*, P. *aerigunosa*, S. *typhi*, and 10 µg/ml against *S. aureus*, *C. albicans* (Kuete *et al.*, 2009).

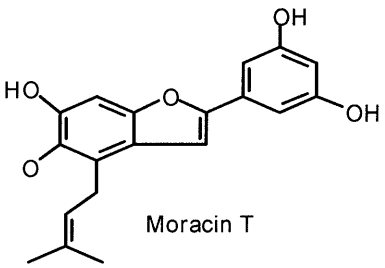
- Isoquercitrin, catechin and epicatechin isolated from *Chiranthodendron pentadactylon* flowers have antisecretory effect on *Vibrio cholerae* toxin induced intestinal fluid accumulation with ID_{50} of 19.2, 51.7 and 8.3 µg/ml against loperamide (positive control) with ID_{50} of 6.1 µg/ml.
- Davidigenin isolated from *Mascarenhasia arborescens* inhibits histamine induced contractile response of rat ileum and Ach induced contractile response on rat duodenum. The compound was reported to have non-competitive concentration dependent inhibitory activity (Desire *et al.*, 2010).
- Vitexin isolated from *Aloysia citriodora* has also been reported to have antispasmolytic activities (Ragone *et al.*, 2007).
- Quercetin has 90.7±0.3% and 79.6±2.3% inhibition of Ach and the depolarized KCl induced contractions on guinea pigs isolated ileum at IC_{50} <0.1 µg/ml respectively (Cimanga *et al.*, 2010).
- Quercitrin was reported to have spasmolytic activities of 82.3±2.3% and 72.1±0.6% against Ach and the depolarized KCl induced contractions on guinea pigs isolated ileum at IC_{50} <0.01 µg/ml respectively (Cimanga *et al.*, 2010).
- Spasmolytic activities of rutin were also reported as 93.4±1.6% and 86.3±2.1% against Ach and the depolarized KCl induced contractions on guinea pigs isolated ileum at IC_{50} <0.01 µg/ml respectively (Cimanga *et al.*, 2010).
- Luteolin isolated from *Pogonatherum crinitum* has iNOS inhibitory activity with E_{max} equals 100.00±0.00% and IC_{50} of 10.41 µM while Kaempferol isolated from the same plant has iNOS inhibitory activity with E_{max} of 95.12±1.15% and the IC_{50} equals 10.61±0.44 µM (Wang *et al.*, 2008)
- Stilbenoids such as r-2-viniferin, trans-amurensin and cis-amurensin isolated from *Vitis amurensis* have LOX inhibitory activity with IC_{50} of 6.39±0.08 µM, 12.1±0.32 µM and 16.3±0.52 µM respectively (Ha *et al.*, 2009).
- Laurentixanthone A isolated from *Vismia laurentii* has good activities with MIC of 4.88 µg/ml against *S. dysenterica, S. flexneri, B. substilis* (Nguemeving *et al.*, 2006).
- 5,7-dihydroxy-2-[14-methoxy-15-propyl phenyl]-4H-chromen-4-one isolated from *Leuca aspera* has superoxide radical scavenging activity of 75.4±0.31% and lipid peroxidation inhibition of 68.7±0.41% at a concentration of 40 ppm (Meghashri *et al.*, 2010).
- Lipid peroxidation inhibitory activity of foeniculoside X isolated from *Foeniculum vulgare* at concentration of 10^{-5}M was reported to be 6.30 nm TBARS/mg LDL (Marino *et al.*, 2007).
- An *in vivo* lipid peroxidation inhibitory activity of arzanol isolated from *Helichrysum italicum* has also been reported (Rosa *et al.*, 2011).



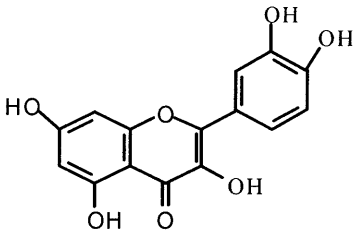
Foeniculosde X



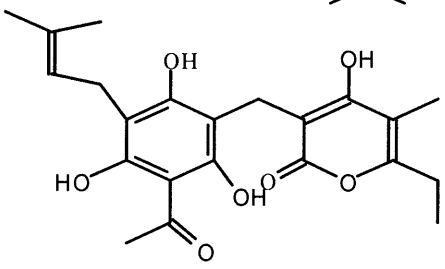
(5, 7-dihydroxy-2-[14-methoxy-15-propyl phenyl]-4H-chromen-4-one



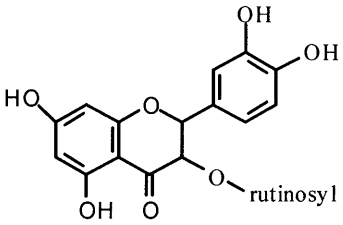
Moracin T



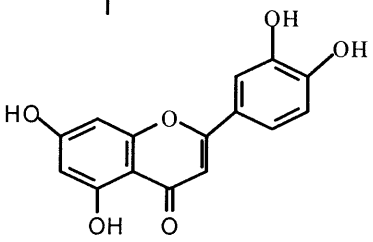
Quercetin



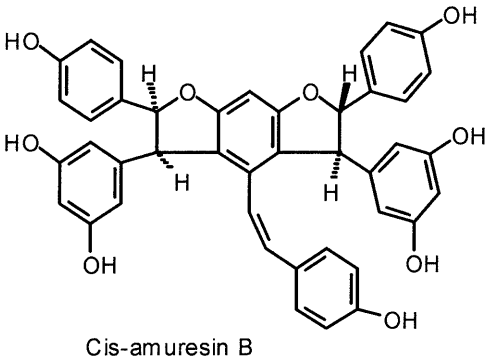
arzanol



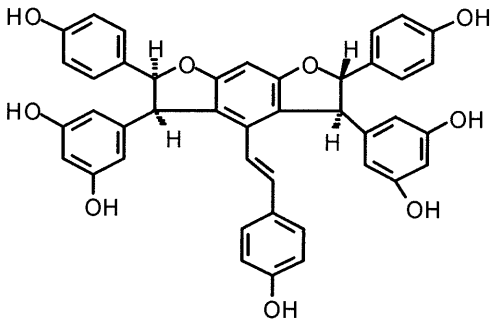
Rutin



Luteolin



Cis-amuresin B



Trans-amuresin B

Fig. 2.12. Chemical structures of bioactive phenolics against diarrhoeal mechanisms

2.16. Ethnobotany and scientific investigation of plant species used traditionally in treating diarrhoea in South Africa

A survey on traditional practice in South Africa indicates that diarrhoea is one of the most prominent ailments being treated with medicinal plants (Dambisya and Tindimwebwa, 2003). At this stage, the scientific validation of their therapeutic potential, standardization, safety and mechanisms of actions of most of the plants is still lacking (Havagiray *et al.*, 2004). Ethnopharmacological properties and phytochemistry of the medicinal plants used for the treatment of diarrhoea in South Africa is reviewed and presented in Appendix 1.

2.17. Conclusion

Many medicinal plants are used in various traditional cultures of South Africa to treat diarrhoea and the associated complications. The traditional recipes include infusions, decoctions, and tinctures of the leaves, stems, roots, seeds and bark of medicinal plants. Several scientific methods have been used to evaluate and validate the traditional use of some of the plants as antidiarrhoeal agents. Such properties investigated as the potential antidiarrhoeal mechanism are the antimotility, antipropulsive, antioxidant, anti-inflammation, antispasmolytic, antimicrobial, antiprotozoal, and immunomodulatory activity of the medicinal plant preparations. Several of these aspects will be examined for some selected medicinal plants in this study.

CHAPTER THREE

Plant selection, collection, extraction and analysis of selected species

3.1. Introduction

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

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

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

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

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

3.2. Solid-liquid extraction

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

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

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

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

3.3. Liquid-liquid fractionation

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

3.4. Thin layer chromatography (TLC)

3.4.1. Phytochemical fingerprints

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

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

3.5. Materials and Methods

3.5.1. Selection of South Africa medicinal plants for antidiarrhoeal screening

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

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

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

3.5.2. Collection of plant materials

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

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

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

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

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

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

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

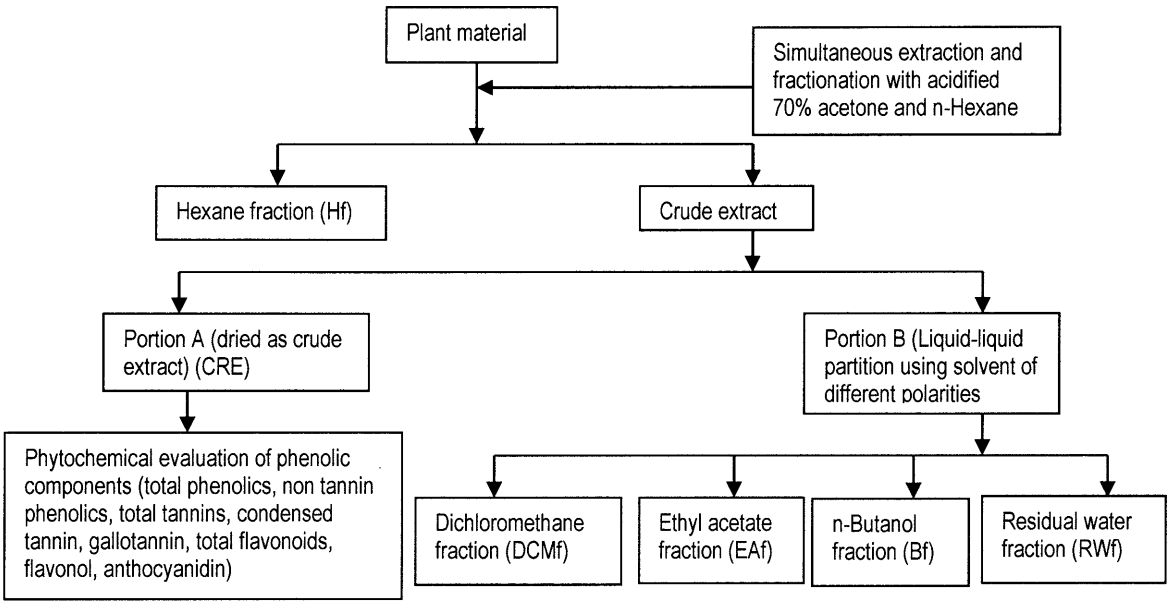


Fig. 3.1. Flow chart for the extraction, phytochemical analysis and fractionation of plant material

3.5.4. Phytochemical profiling

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

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

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

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

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

3.6. Quantification of the phenolic constituents of the extracts

3.6.1. Determination of total phenolic constituents

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

3.6.2. Determination of total tannin

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

3.6.3. Determination of proanthocyanidin

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

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

3.6.4. Determination of condensed tannin

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

3.6.5. Determination of hydrolysable tannin (gallotannin)

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

3.6.6. Determination of total flavonoids and flavonol

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

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

3.6.7. Determination of anthocyannin

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

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

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

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

3.7. Results

3.7.1. Yield of extractions and fractionations processes

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

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

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

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

3.7.2. Phytochemical screening (fingerprints)

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

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

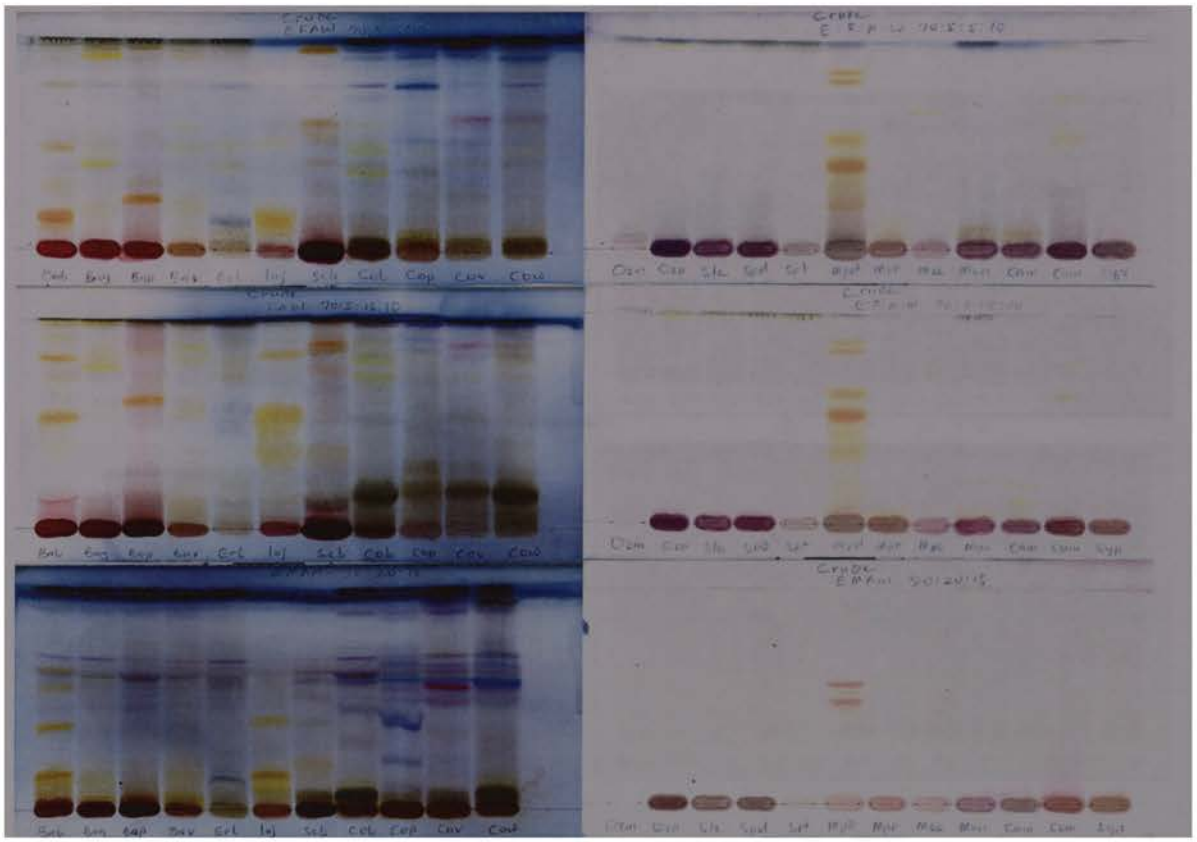


Fig.3.2. Chromatograms of 100 µg of crude extracts of different plant species developed with ethyl acetate: acetic acid: formic acid: water (75:5:5:10) (top), ethyl acetate: acetic acid: formic acid: water (70:5:15:10) (middle) and ethyl acetate: methanol: ammonia (90:20:15) (bottom) and visualized with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

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

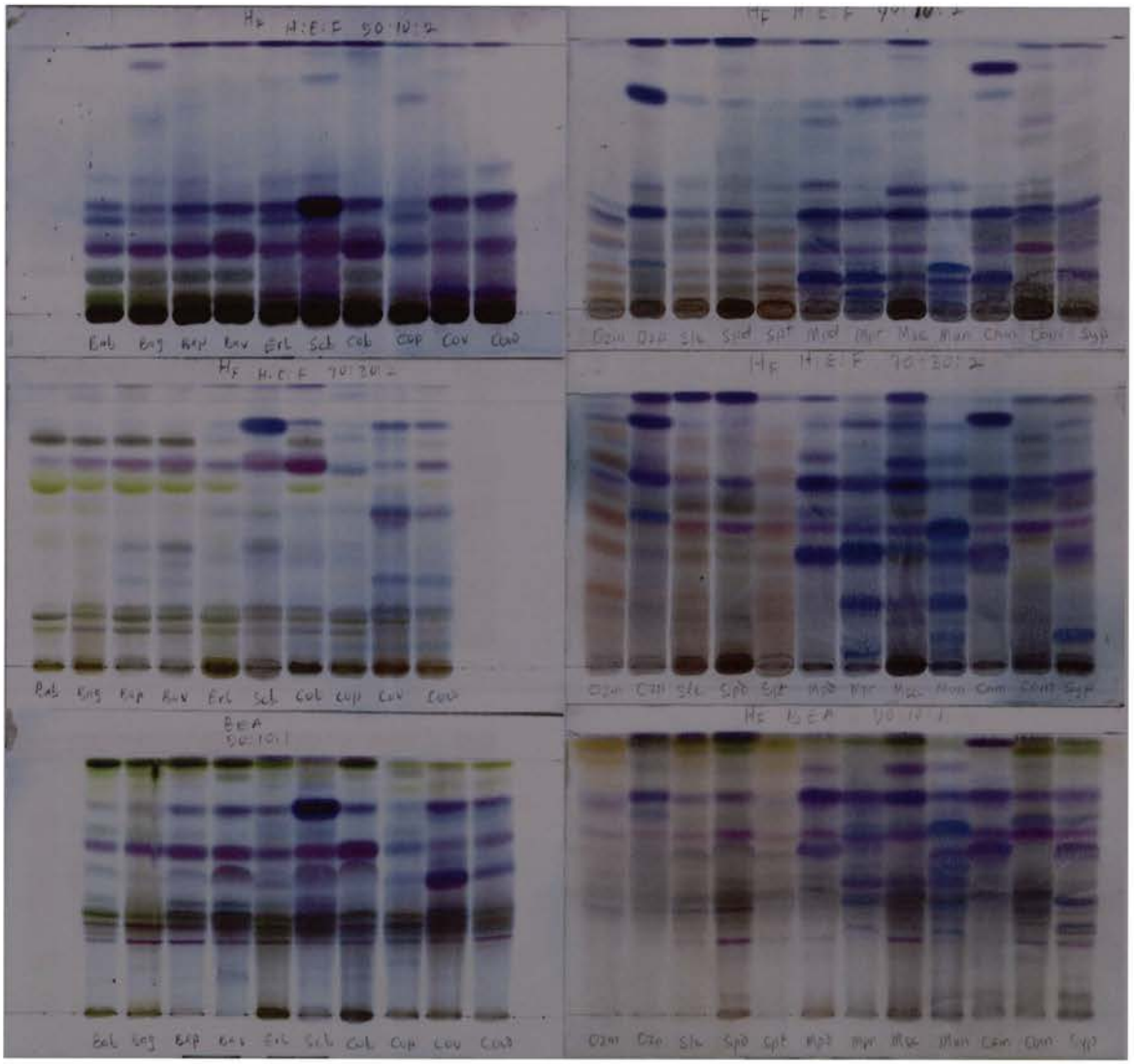


Fig. 3.3: Chromatograms of the hexane fractions of different plant species developed with hexane: ethyl acetate: formic acid (90:10:2) (top), hexane: ethyl acetate: formic acid (70:30:2) (middle), and benzene: ethyl acetate: ammonia (90:10:1) (bottom) and visualized with vanillin sulphuric acid. For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

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

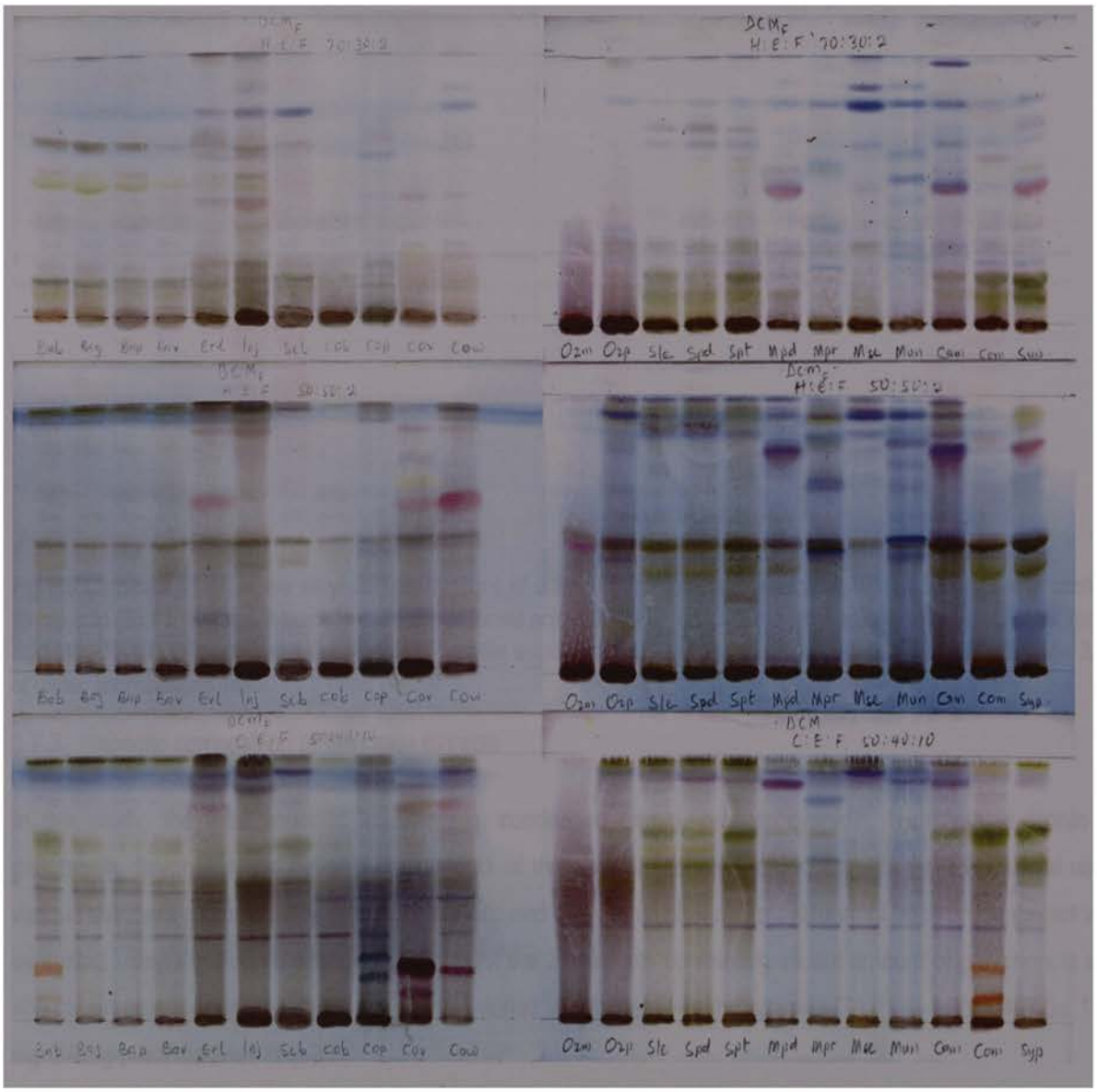


Fig 3.4: Chromatograms of the dichloromethane fractions of different plant species developed with hexane: ethyl acetate: formic acid (70:30:2) top, hexane: ethyl acetate: formic acid (50:50:2) (middle) and chloroform: ethyl acetate: formic acid (50:40:10) bottom and visualized with vanillin sulphuric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

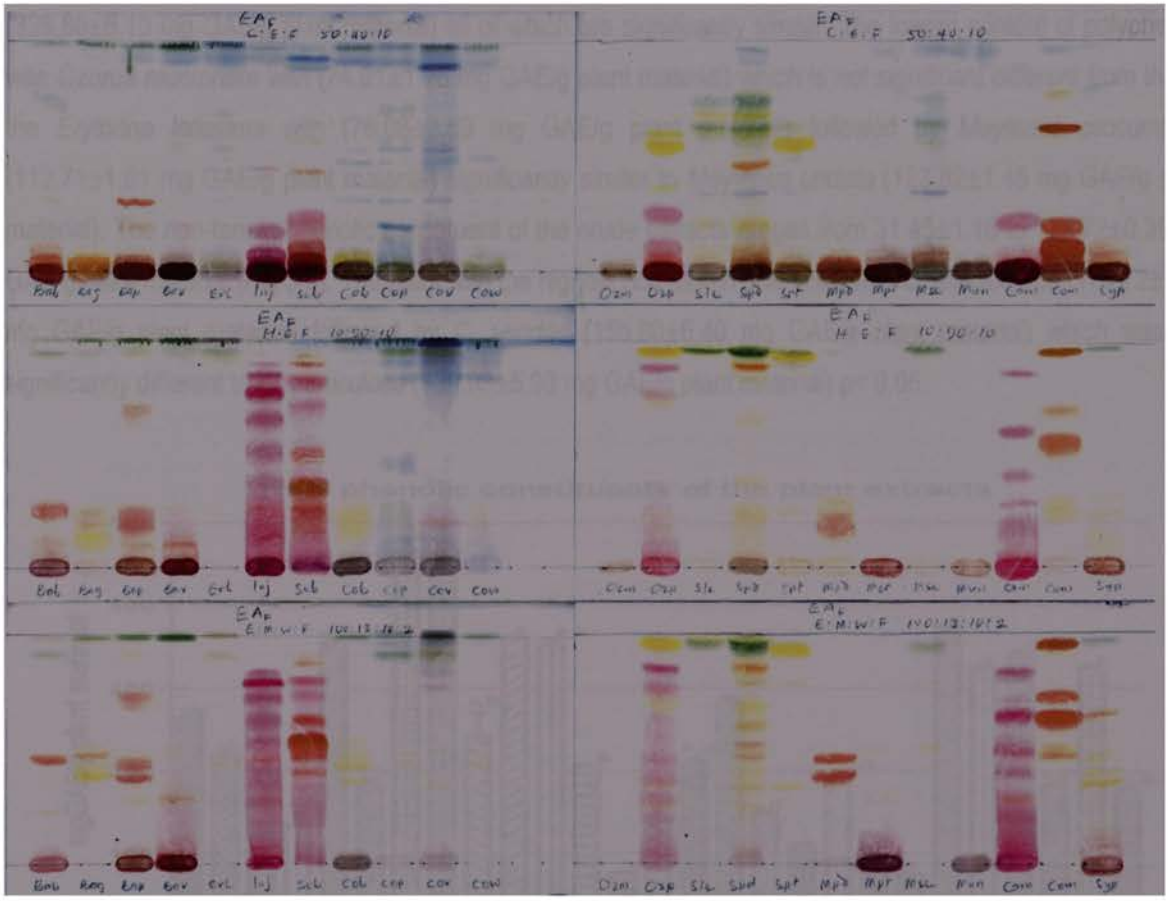


Fig 3.5 Chromatograms of the ethyl acetate fractions of different plant species developed with chloroform: ethyl acetate: formic acid (50:40:10) (top), hexane: ethyl acetate: formic acid (10:90:10) (middle) and ethyl acetate: methanol: water: formic acid (100:13:10:2) (bottom) and visualized with vanillin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

3.7.3. Phenolic composition of the crude extracts

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

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

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

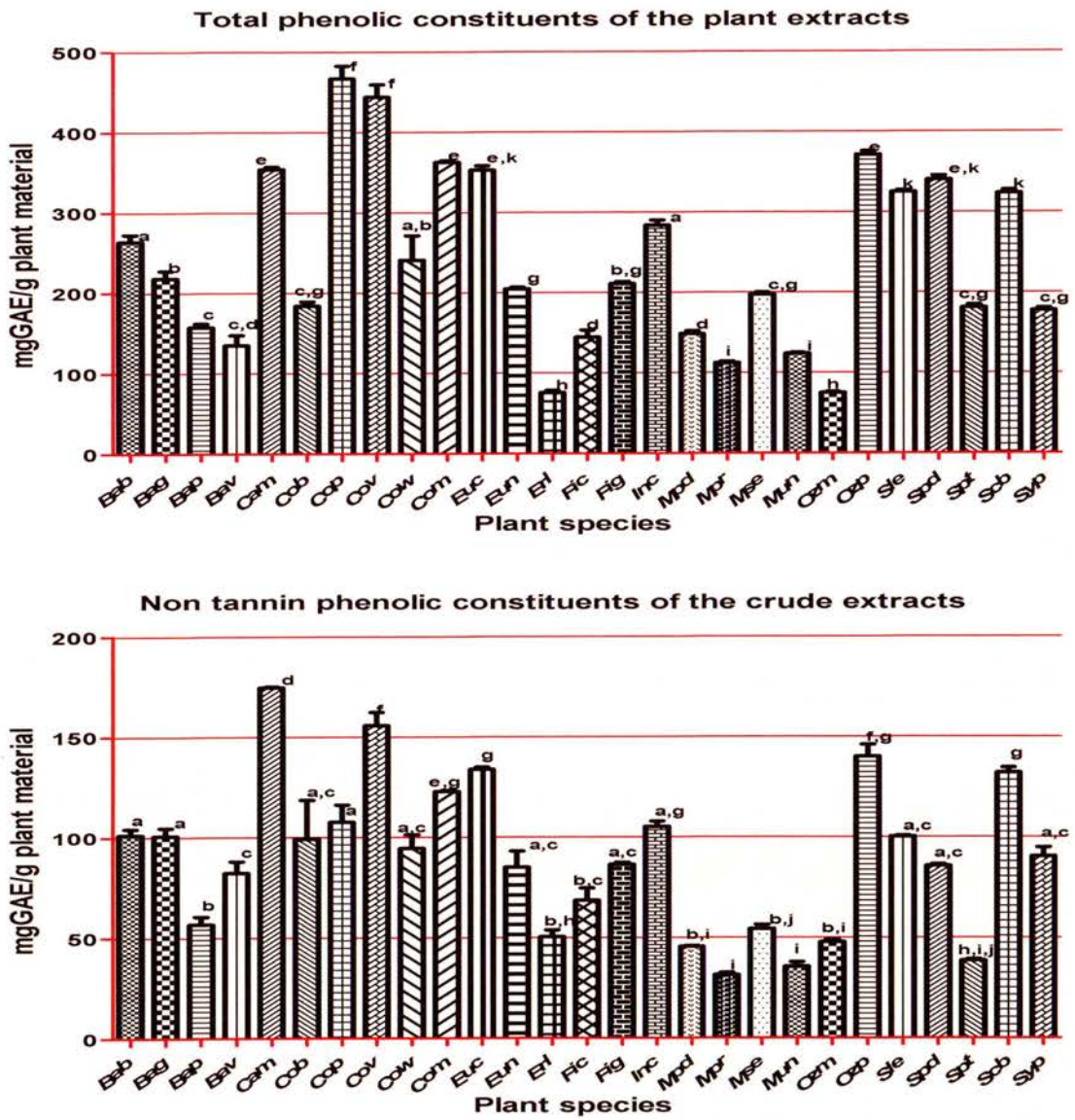


Fig. 3.6. Total phenolic and non-tannin constituent of the crude plant extracts

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

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

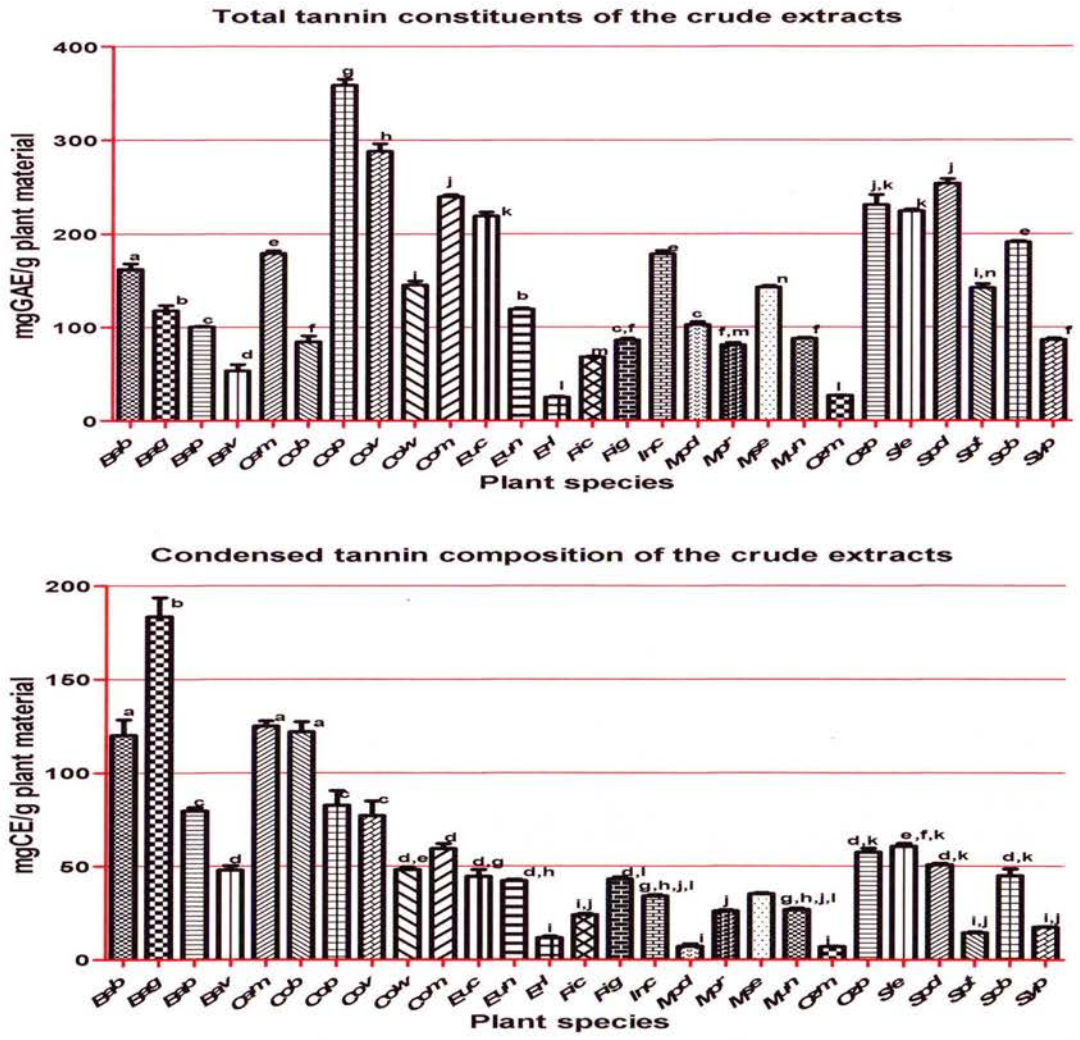


Fig. 3.7. Total tannin and condensed tannin of the crude extracts

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

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

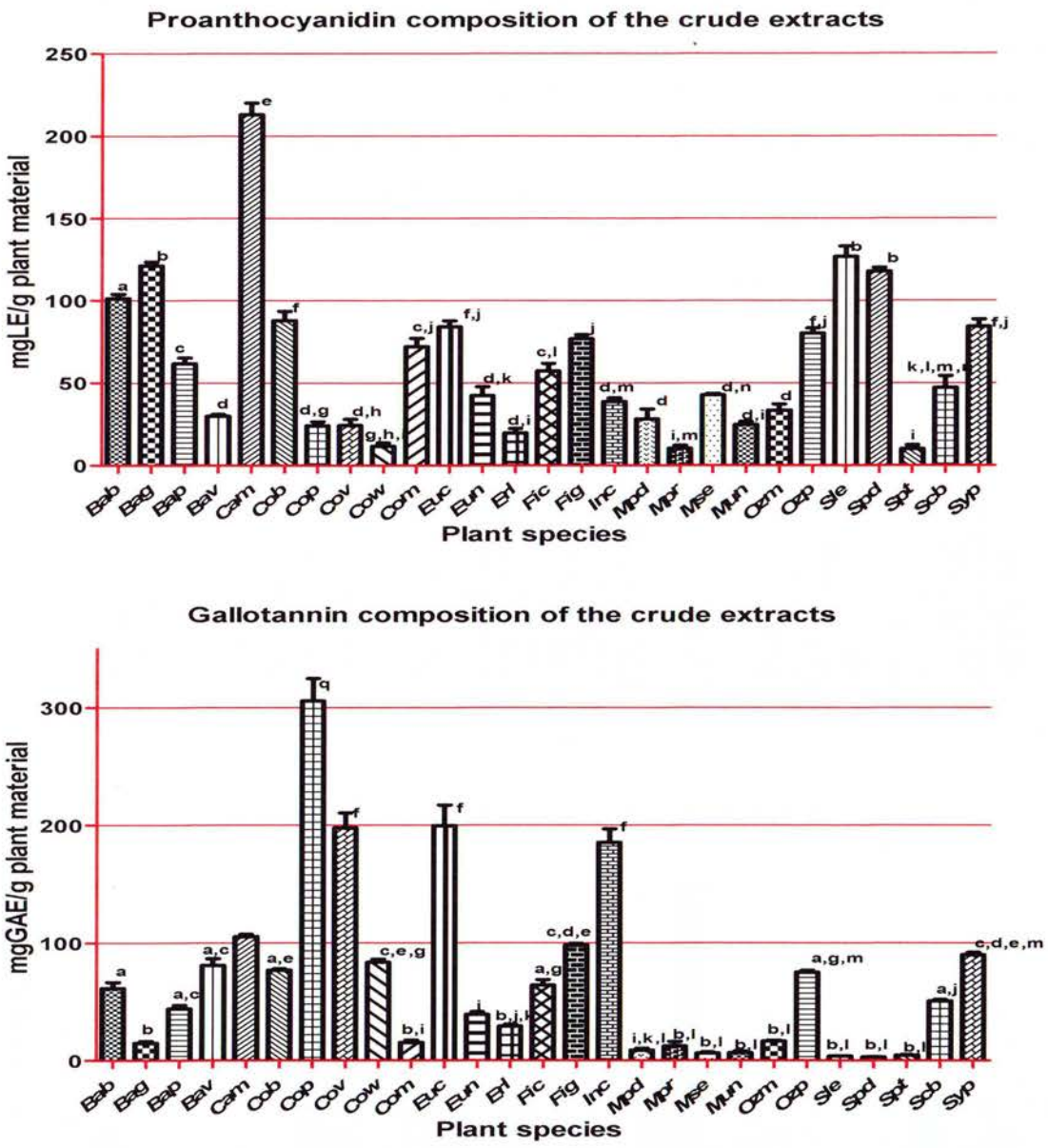


Fig. 3.8. Proanthocyanidin and gallotannin constituent of the crude extracts

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

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

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

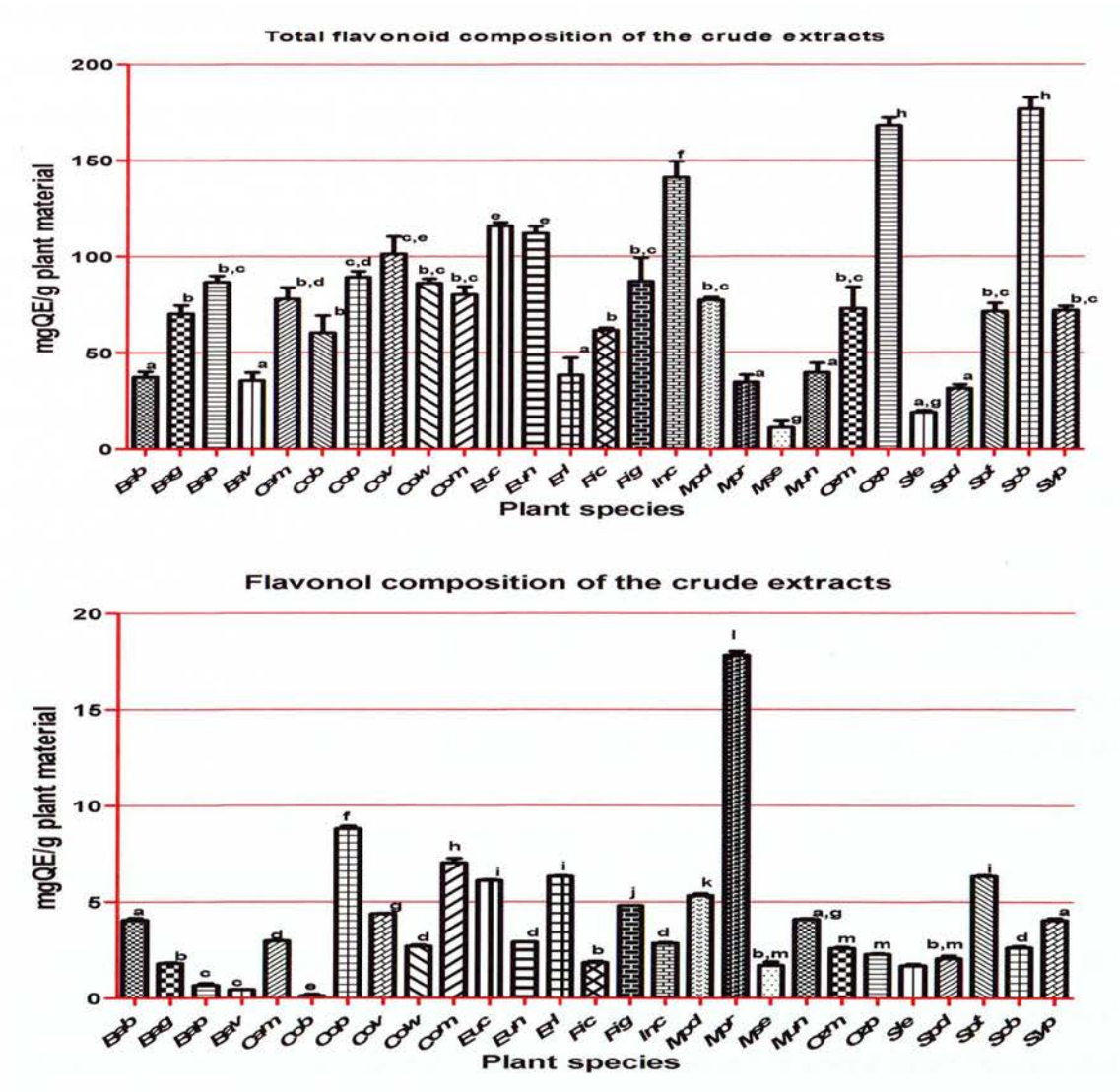


Fig. 3.9. Total flavonoids and the flavonol constituent of the crude extracts

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

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

3.8. Discussion

3.8.1. Yield

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

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

3.8.2. Thin layer chromatogram

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

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

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

3.8.3. Phenolic constituents of the crude extracts

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

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

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

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

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

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

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

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

3.9. Conclusion

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

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

CHAPTER FOUR

Antimicrobial activities of the plant extracts against potential diarrhoeal pathogens

4.0. Introduction

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

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

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

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

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

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

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

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

4.1. Qualitative antimicrobial (Bioautography) assay

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

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

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

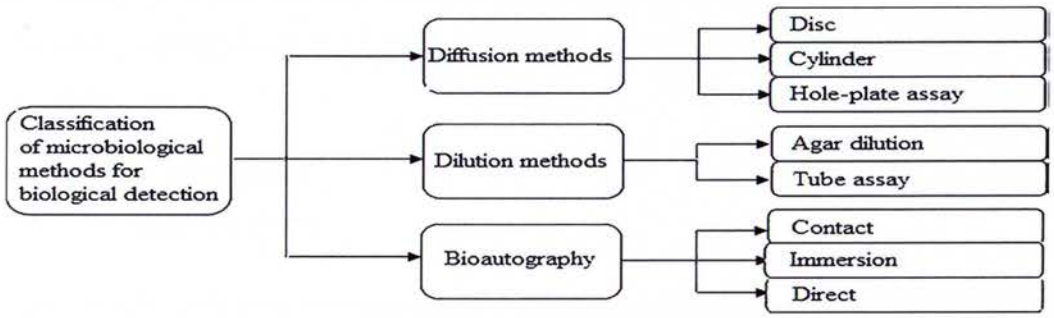


Fig 4.1. The classification of microbiological methods for biological detection (Adopted from Choma and Grzelak, 2011)

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

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

4.3. Selection of microorganisms used in the study

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

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

4.4. Material and Methods

4.4.1. Microorganism strains

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

4.4.2. Culturing of the Bacteria

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

4.4.3. Bioautography against some pathogenic microorganisms

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

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

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

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

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

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

4.5. Results

4.5.1. Microbial bioautography

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

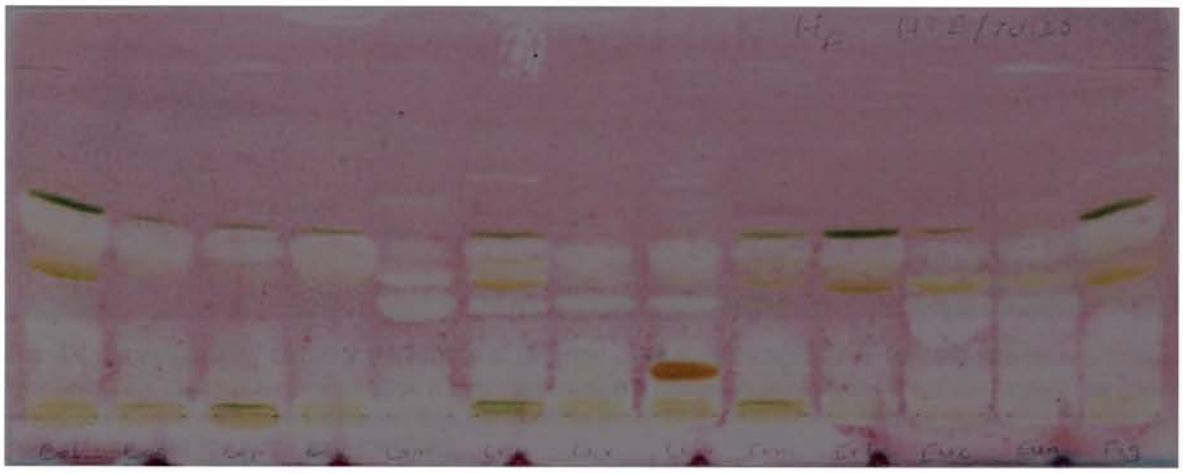


Fig. 4.2. Bioautography of hexane (upper) fraction of different plant species against *S. aureus* (Bab (*Bauhinia bowkeri*), Bag (*Bauhinia galpinii*), Bap (*Bauhinia petersiana*),Cam (*Carissa macrocarpa*), , Cop (*Combretum padoides*), Cov (*Combretum vendae*), Cow (*Combretum woodii*), Com (*Commiphora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

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

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

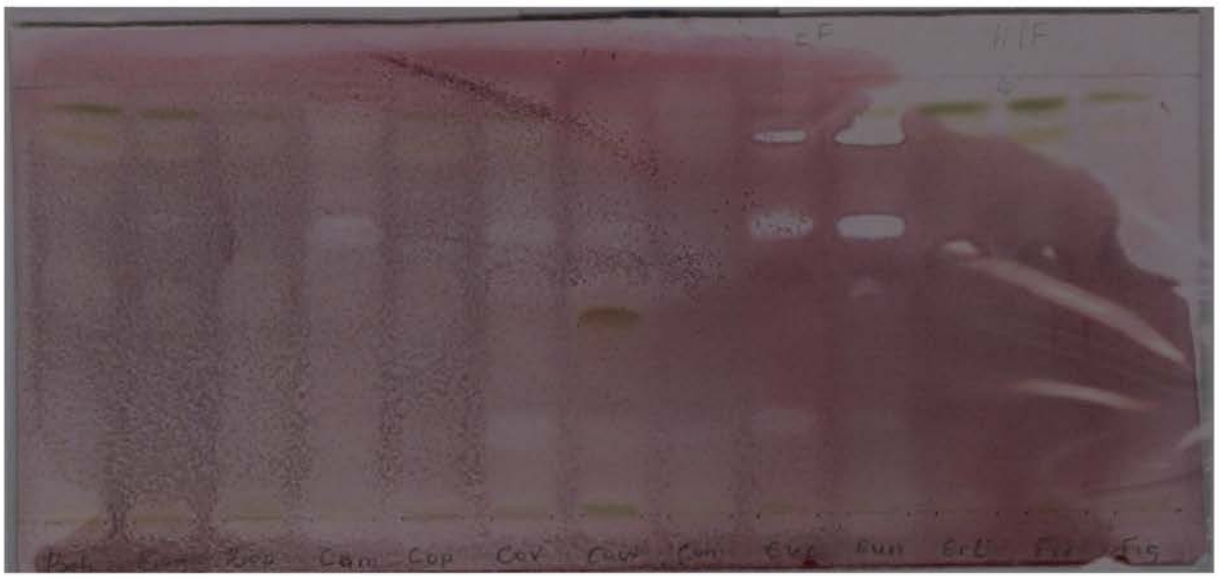


Fig. 4.4. Bioautography of hexane fractions of different plant species against *E. faecalis* (Bab (*Bauhinia bowkeri*), Bag (*Bauhinia galpinii*), Bap (*Bauhinia petersiana*),Cam (*Carissa macrocarpa*), Cop (*Combretum padoides*), Cov (*Combretum vendae*), Cow (*Combretum woodii*), Com (*Commiphora harveyi*),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

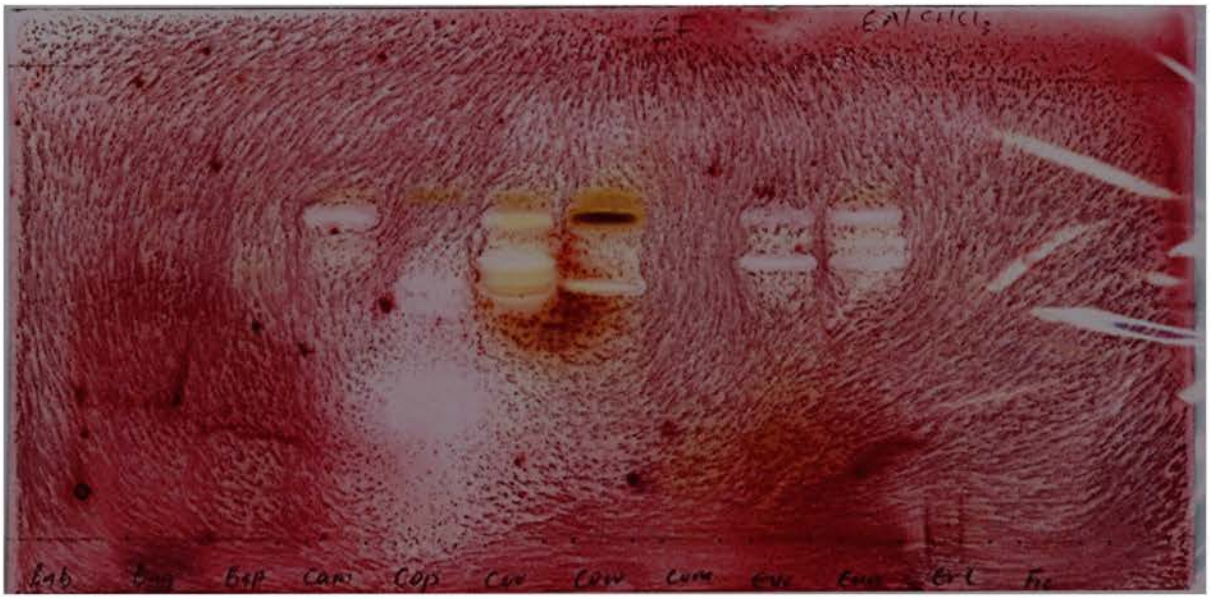


Fig. 4.5. Bioautography of dichloromethane fractions of different plant species against *E. coli* (Bab (*Bauhinia bowkeri*), Bag (*Bauhinia galpinii*), Bap (*Bauhinia petersiana*),Bav (*Bauhinia variegata*) Cam (*Carissa macrocarpa*), , Cop (*Combretum padoides*), Cov (*Combretum vendae*), Cow (*Combretum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

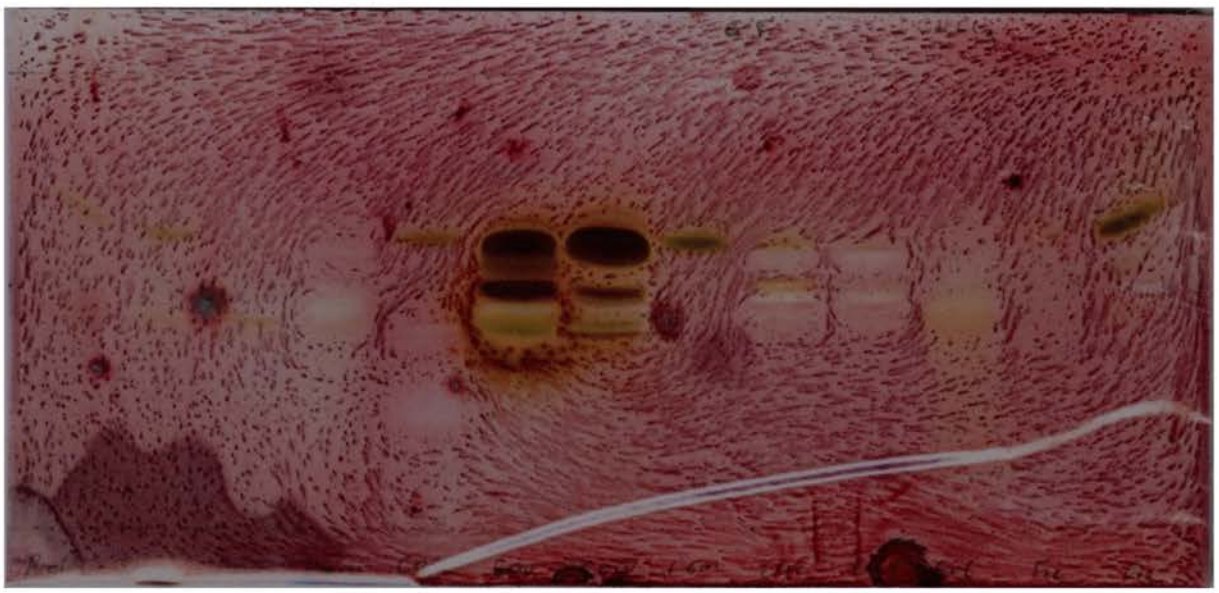


Fig. 4.6. Bioautography of dichloromethane fractions of different plant species against *E. faecalis* (Bab (*Bauhinia bowkeri*), Bag (*Bauhinia galpinii*), Bap (*Bauhinia petersiana*),Bav (*Bauhinia variegata*) Cam (*Carissa macrocarpa*), , Cop (*Combretum padoides*), Cov (*Combretum vendae*), Cow (*Combretum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

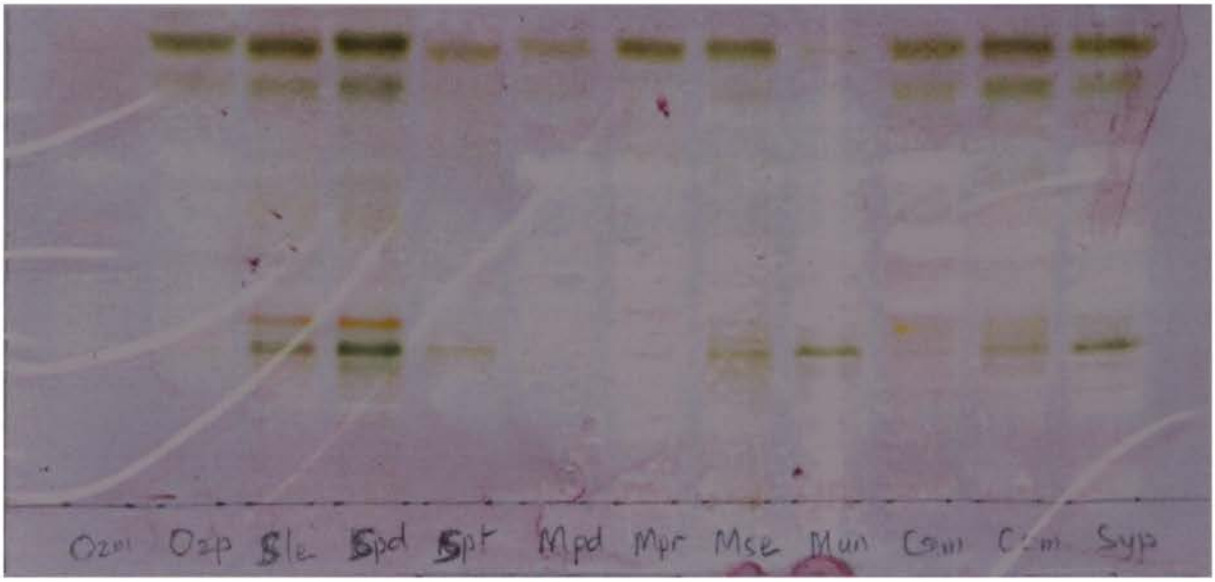


Fig. 4.7. Bioautography of hexane of different plant speicies against *C. neoformans* (Ozm (*Ozoroa mucronata*), Ozp (*Ozoroa paniculosa*), Sle (*Searsia leptodictya*), Spd (*Searsia pendulina*), Spt (*Searsia pentheri*), Mpd (*Maytenus peduncularis*), Mpr (*Maytenus procumbens*), Mse (*Maytenus senegalensis*),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

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

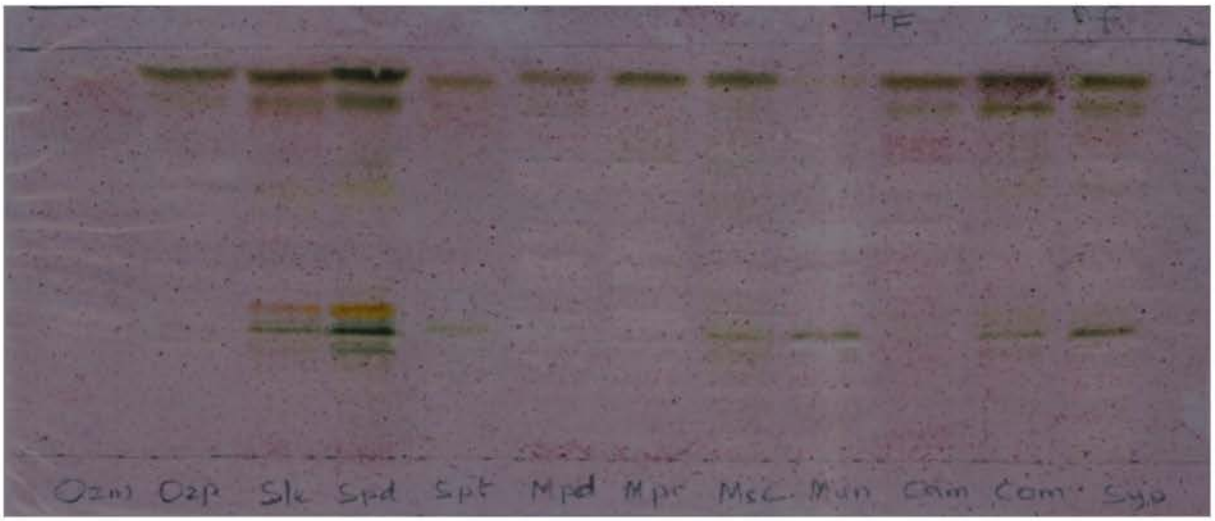


Fig. 4.9. Bioautography of hexane fractions against *A. fumigatus* (Ozm (*Ozoroa mucronata*), Ozp (*Ozoroa paniculosa*), Sle (*Searsia leptodictya*), Spd (*Searsia pendulina*), Spt (*Searsia pentheri*), Mpd (*Maytenus peduncularis*), Mpr (*Maytenus procumbens*), Mse (*Maytenus senegalensis*), Mun (*Maytenus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

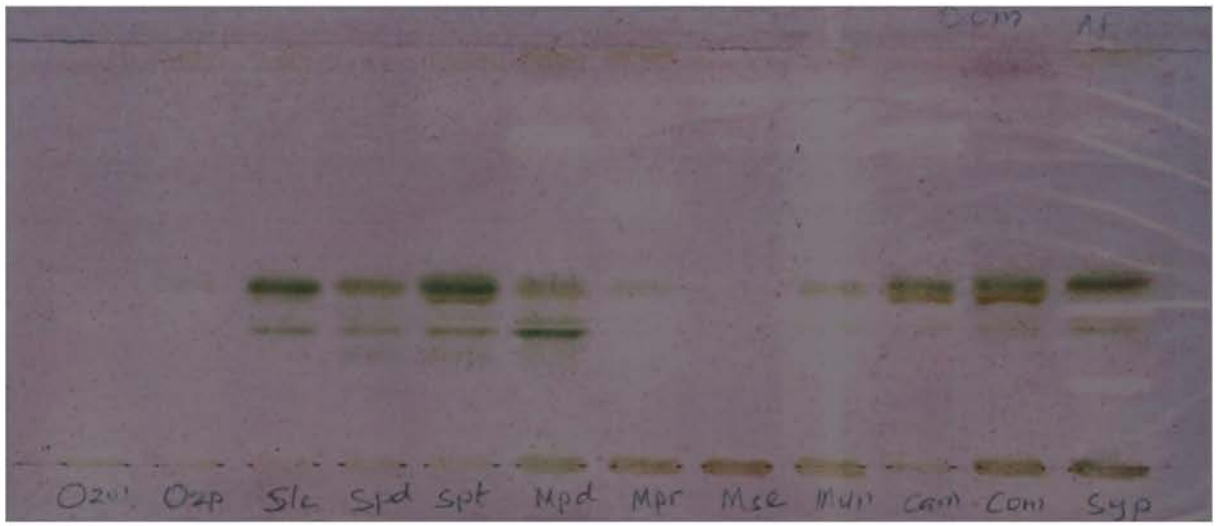


Fig. 4.10. Bioautography of dichloromethane fractions against *A. fumigatus* (Ozm (*Ozoroa mucronata*), Ozp (*Ozoroa paniculosa*), Sle (*Searsia leptodictya*), Spd (*Searsia pendulina*), Spt (*Searsia pentheri*), Mpd (*Maytenus peduncularis*), Mpr (*Maytenus procumbens*), Mse (*Maytenus senegalensis*),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

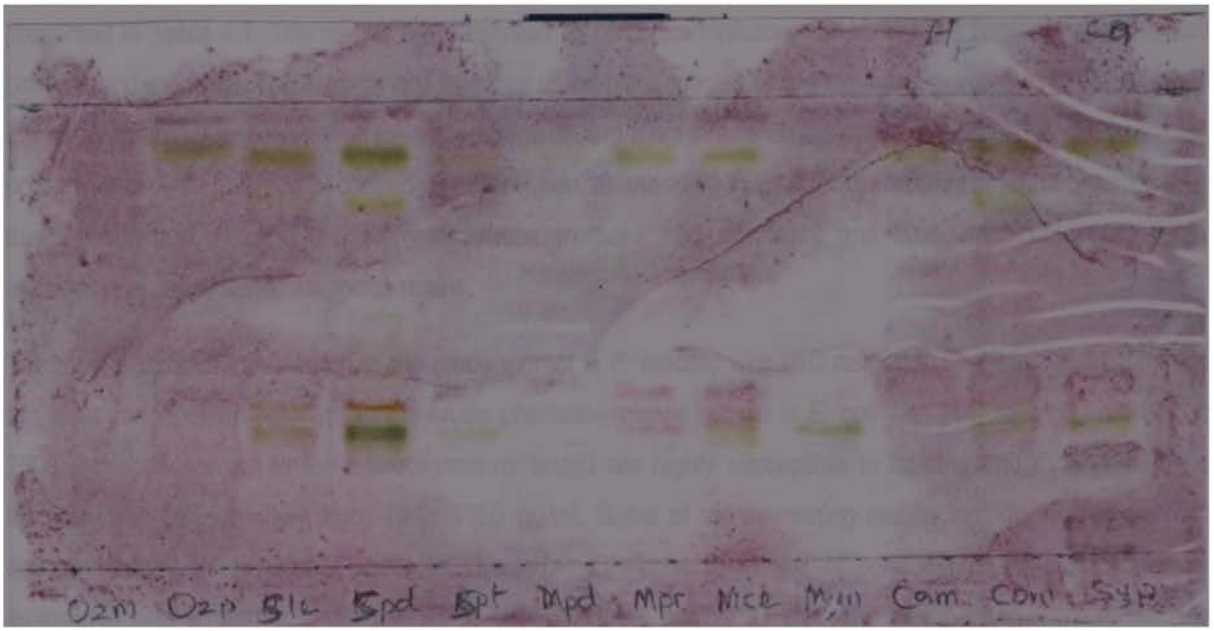


Fig. 4.11. Bioautography of hexane fractions against *C. albicans* (Ozm (*Ozoroa mucronata*), Ozp (*Ozoroa paniculosa*), Sle (*Searsia leptodictya*), Spd (*Searsia pendulina*), Spt (*Searsia pentheri*), Mpd (*Maytenus peduncularis*), Mpr (*Maytenus procumbens*), Mse (*Maytenus senegalensis*), Mun (*Maytenus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

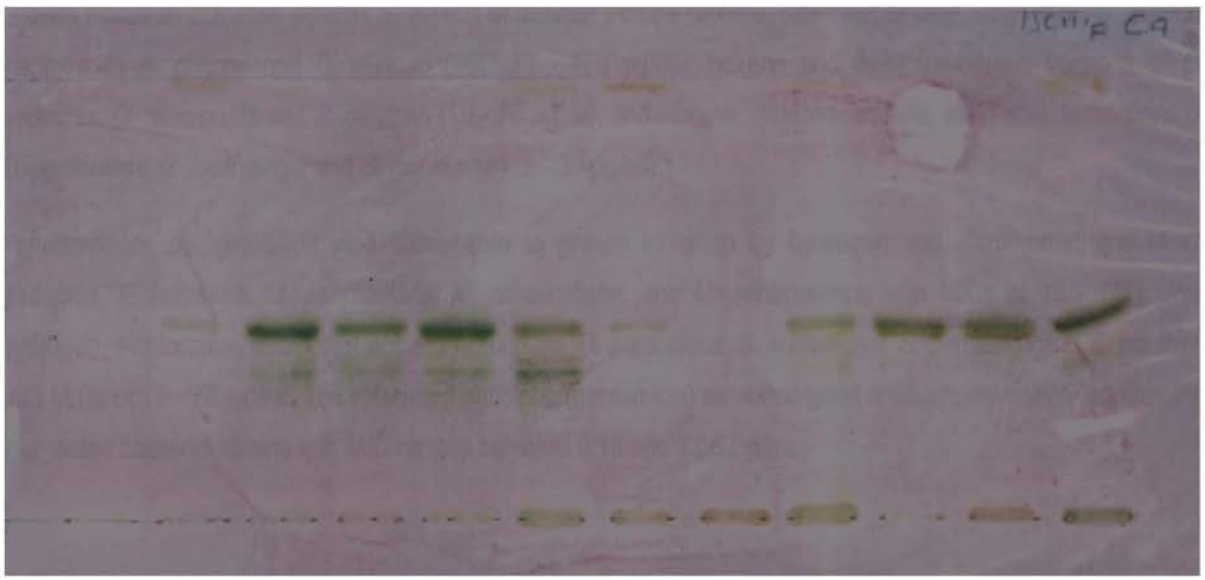


Fig. 4.12. Bioautography of dichloromethane fractions against *C. albicans* (Ozm (*Ozoroa mucronata*), Ozp (*Ozoroa paniculosa*), Sle (*Searsia leptodictya*), Spd (*Searsia pendulina*), Spt (*Searsia pentheri*), Mpd (*Maytenus peduncularis*), Mpr (*Maytenus procumbens*), Mse (*Maytenus senegalensis*),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

4.5.2. Minimum inhibitory concentration (MIC)

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

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

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

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

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

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

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

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

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

4.5.3. Minimum inhibitory concentration (MIC)

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

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

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

4.6. Discussion

4.6.1. Antimicrobial bioautography

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

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

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

4.6.2. Minimum inhibitory concentration (MIC)

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

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

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

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

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

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

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

4.7. Conclusion

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

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

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

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

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

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).

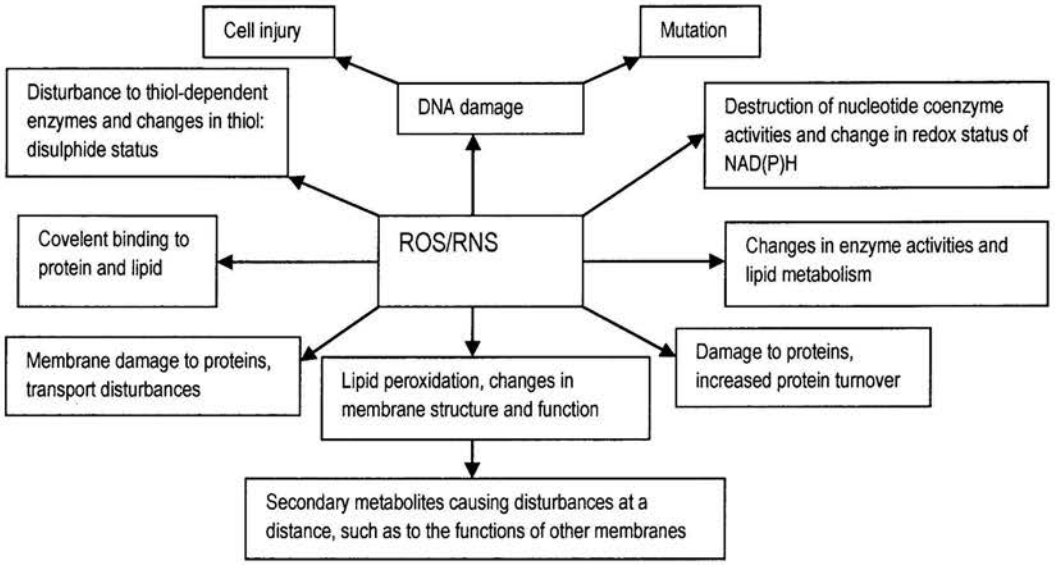


Fig. 5.1. Deleterious reactions from the production of reactive free radicals in biological system (amended from Slater *et al.*, 1987)

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.

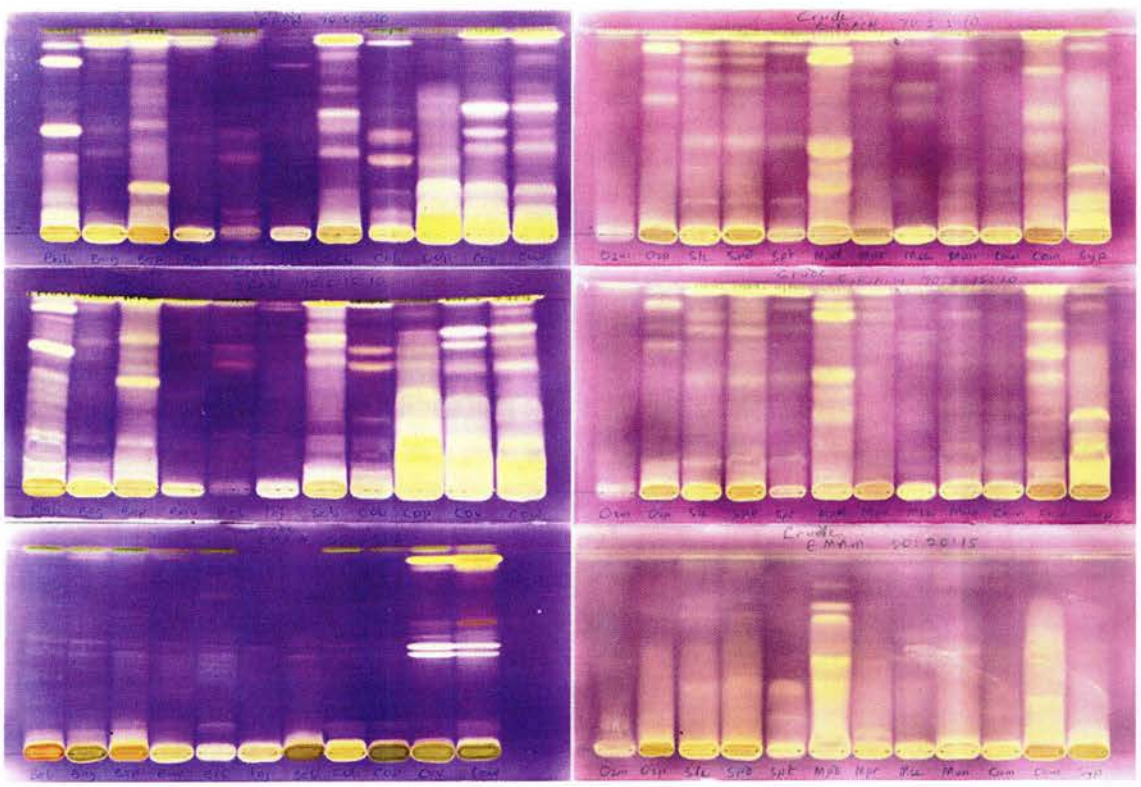


Fig.5.2. TLC-DPPH profiles of the crude extracts of extracts of different plants (left to right **Bab** (*Bauhinia bowkeri*), **Bag** (*Bauhinia galpinii*), **Bap** (*Bauhinia petersiana*), **Bav** (*Bauhinia variegata*), **Erl** (*Erythrina latissima*), **Inc** (*Indigofera cylindrica*), **Scb** (*Schotia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

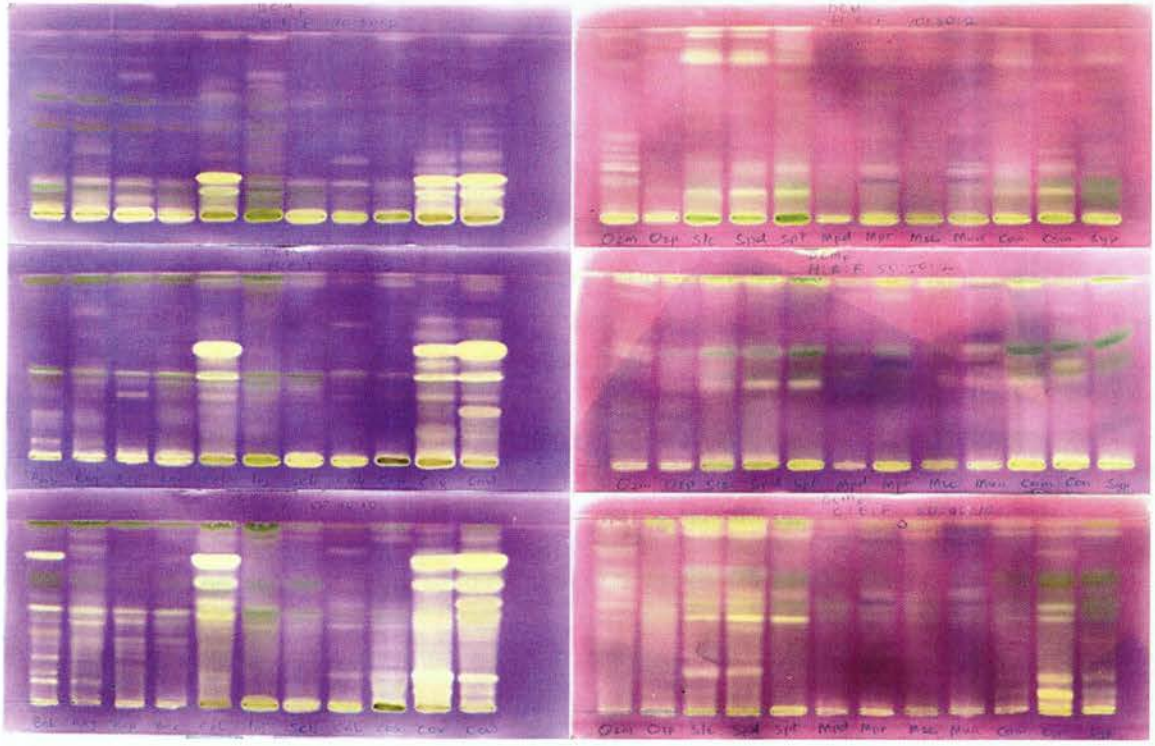


Fig.5.4 TLC-DPPH profiles of the dichloromethane fractions of different plants (left to right **Bab** (*Bauhinia bowkeri*), **Bag** (*Bauhinia galpinii*), **Bap** (*Bauhinia petersiana*), **Bav** (*Bauhinia variegata*), **Erl** (*Erythrina latissima*), **Inc** (*Indigofera cylindrica*), **Scb** (*Schotia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

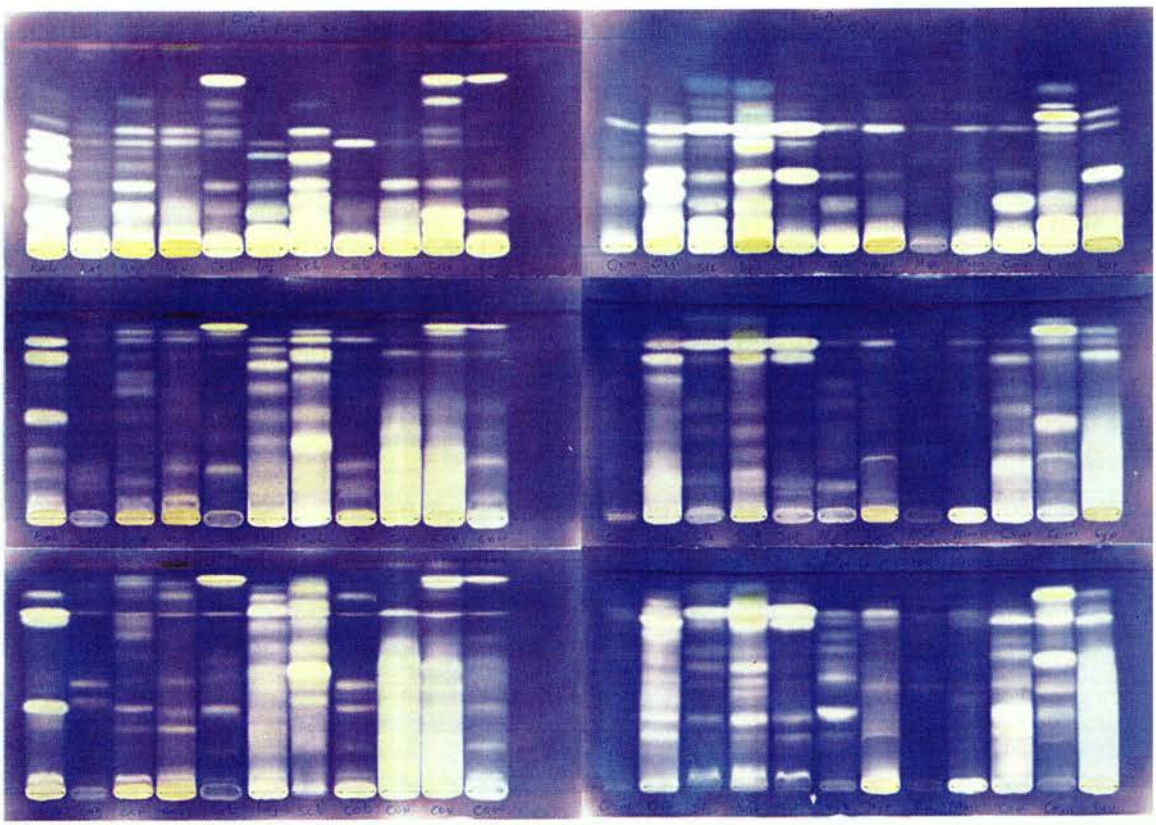


Fig. 5.5. TLC-DPPH profiles of the ethyl acetate fractions of different plants left tor right (left to right **Bab** (*Bauhinia bowkeri*), **Bag** (*Bauhinia galpinii*), **Bap** (*Bauhinia petersiana*), **Bav** (*Bauhinia variegata*), **Erl** (*Erythrina latissima*), **Inc** (*Indigofera cylindrica*),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

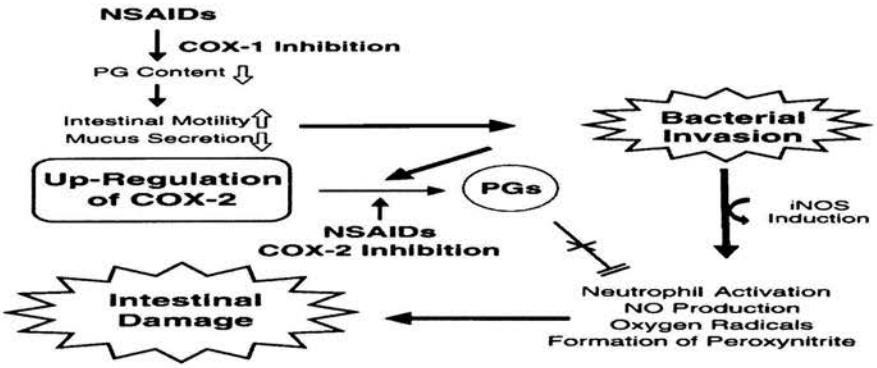


Fig. 6.1. Roles of COX-1 and COX-2 in the pathogenic mechanism of NSAID-induced intestinal damage (Takeuchi et al, 2010)

- 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).

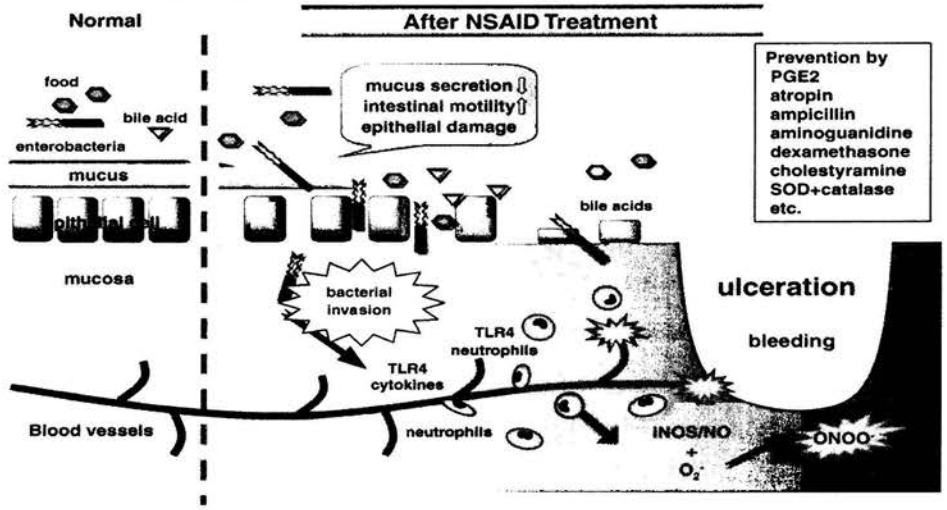


Fig. 6.2. Various factors involved in the pathogenesis of indomethacin-induced small intestinal lesions (Takeuchi et al, 2010)

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.

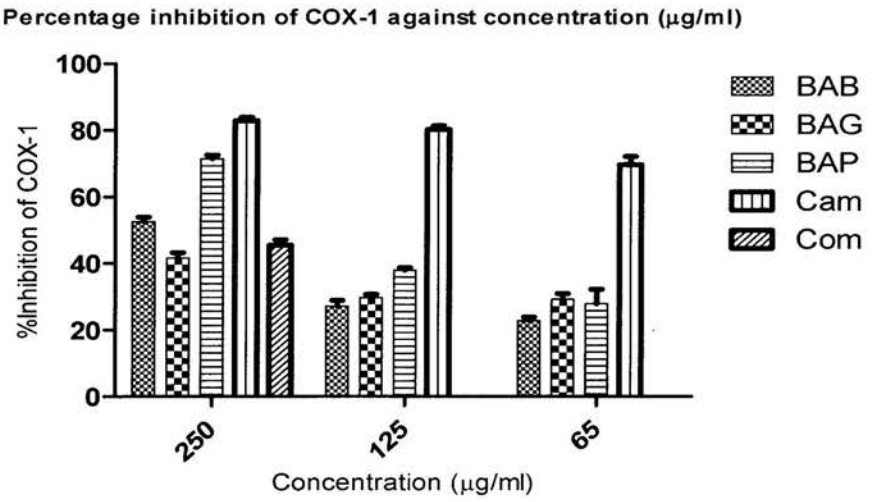


Fig. 6.3. COX-1 inhibitory activity of some selected phenolic-enriched crude extracts

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.

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 anti-infectious, anti-inflammatory, antioxidant, antidiarrhoea and anti-parasitic 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 temperature of 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±2.03 µg/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	LD_{50} (µg/ml)
Bauhinia bowkeri	17.90±2.56
Bauhinia galpinii	35.68±2.15
Bauhinia petersiana	40.68±18.13
Bauhinia variegata	76.37±7.50
Combretum bracteosum	48.81±6.15
Combretum padoides	9.03±0.20
Combretum vendae	5.70±1.25
Combretum woodii	3.51±2.03
Euclea crispa	31.61±4.04
Euclea natalensis	26.99±4.48
Maytenus peduncularis	89.41±16.37
Maytenus procumbens	187.71±19.92
Maytenus senegalensis	87.62±3.03
Maytenus undata	99.17±11.88
Ozoroa mucronata	741.90±44.22
Ozoroa paniculosa	16.58±1.85
Searsia leptodictya	25.09±2.40
Searsia pendulina	22.30±2.42
Searsia pentheri	50.62±4.30

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.

CHAPTER EIGHT

Motility modulation potential of *Bauhinia galpinii* and *Combretum vendae* phenolic-enriched leaf extracts on isolated rat ileum

8.0. Introduction

Gastrointestinal tract (GIT) used the smooth muscle of the mucosal lining enriched with an enteric neural network to regulate propulsive transport and mixing of food material directionally through the digestive systems (Wood, 2004). The neural network initiates and coordinates secretion and absorption across the intestinal lumen as well (Bohn and Raehal, 2006).The enteric neurons function independent of the central nervous system (CNS), therefore referred to as enteric nervous system (ENS). Enteric nervous system controls the motility and contractility of the GIT as its rate and intensity of contraction regulates the absorption of fluid, and expulsion of solid material. Therefore ENS exhibit significant role in GIT disorders such as diarrhoea and constipation through these means. Neurotransmitters such as acetylcholine (ACh), serotonin (5-hydroxytryptamine (5-HT)), substance P, histamine and opioids are the important chemical mediators in contractile regulatory actions of ENS (Farthing, 2002). The activities of the neurotransmitters in the intestine are coordinated by a large number of receptors and sub-receptors. Some of the receptors have been proved to play essential roles in GIT disorders such as peristaltic colonic motility, diarrhoeal and constipation diseases.

Some of the diarrhoea aetiologies such as infectious pathogens or their toxins, inflammatory mediators and oxidation by-products targets to control the peristaltic colonic movement by manipulating the ENS, and also control fluid and electrolyte movement across the intestinal mucosa (Guttman and Finlay, 2008). The modulations in the quantity of the neurotransmitters or the activity of the receptors can have enormous effects on intestinal motility and contraction. The process may help in regulating absorption or secretion of fluid and electrolyte by the intestine; hence provide relief against GIT disorders including diarrhoea and constipation diseases (Sikander *et al*., 2009).

Enteric nervous system presents an attractive potential target for pharmacological intervention in diarrhoea. The use of agonists and antagonist that target these ENS hormone receptors are routinely used clinically to modulate intestinal motility, absorption and secretion. Antispasmodic or antimotility (atropine, clonidine and deodorized tincture opium), and antisecretory agents (racecadotril, octreotide) are used to treat or prevent smooth muscle contraction and control intestinal secretion, thus alleviating many symptoms of GIT disorder including diarrhoea. However, prolonged uses of these drugs are often associated with some side effects such as dry mouth and urinary retention for antimuscarinic drugs, headache, nausea, vomiting and constipation for calcium blockers. Several medicinal plants are used by different traditional cultures across the world in alleviating GIT disorders

clinically manifesting as diarrhoea without reported cases of adverse effects. These provide the rationale in continuous search for safer and efficient drugs from plant phytochemicals that might target a specific receptor.

In South Africa and other developing countries treatment of gastrointestinal disorders such as diarrhoea with medicinal plants are particularly common in rural areas. The antidiarrhoea activities of medicinal plant extracts can be exhibited through spasmolytic effects (intestinal smooth muscle relaxation), delay gastrointestinal transit, suppress gut motility, stimulate water absorption or reduce electrolyte secretion. In contrast, the mechanism of actions of medicinal plants used in constipation include spasmogenic effects (intestinal smooth muscle contraction), rapid gastrointestinal transit, activated gut motility, suppressed water absorption or increase electrolyte secretion (Gilani *et al*, 2005a). All these effects are related to the regulation of ENS motility and contractility. However, scientific evaluations of the therapeutic claims as well as mechanisms of action are still unreported for many of the antidiarrhoeal plants used in traditional medicine. The aim of this study therefore is to evaluated motility regulatory potentials and determined possible mechanism of action of phenolic-enriched leaf of *Bauhinia galpinii* and *Combretum vendae* as antidiarrhoea medicinal plants on isolated rat ileum.

8.1. Drugs and reagents

Acetylcholine hydrochloride (Ach), serotonin (5-HT), nicotine, Histamine, Prostaglandin E_2 (PGE_2), Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), N^G-nitro-l-arginine methyl ester (L-NAME), Carbachol, Pilocarpine, , Cyclopiazonic acid, Dimethylsulphoxide (DMSO), Sodium chloride (NaCl), Potassium chloride (KCl), Calcium chloride ($CaCl_2$), Sodium bicarbonate ($NaHCO_3$), Magnesium sulphate ($MgSO_4$), Potassium hydrogen phosphate (KH_2PO_4), Glucose, and carbogen

8.2. Animals

Male Wistar rats (250-300 g) obtained from University of Pretoria Biological Research Centre (UPBRC), Faculty of Veterinary Science, Onderstepoort, Pretoria were used. All animals were housed under standard environmental conditions and provided with food and water ad libitum. All the procedures were in accordance with the guidelines for use of experimental animals established by the Animal Use and Care Committee (AUCC), University of Pretoria based on specification in the South African National Standard (SAN 10386-2008). The approval of ethical committee at Faculty of Veterinary Science, University of Pretoria was obtained before the start of the work. The project was also approved by Faculty of Veterinary Science, University of Pretoria research committee (UP-RESCOM) with approval number of V027-10.

8.2.1. Isolated ileum preparation

The animal was humanely sacrificed with inhalation of isoflurane and dissected immediately. The ileum was removed and placed in carbogenated (95% O_2 and 5% CO_2) Krebs solution with the following composition (g/l): NaCl, 6.94; KCl, 0.354; KH_2PO_4, 0.163; $NaHCO_3$, 2.1; $MgSO_4$, 0.370; $CaCl_2$, 0.367; glucose, 2.07 and pH 7.4. The intestinal content was removed by washing with Kreb's solution and the mesenteric constituents were eliminated. Longitudinal segments (1.5–2.0 cm) obtained from the distal ileum were placed in a 50 mL thermostatically controlled (37°C) organ bath containing Krebs solution gassed with carbogen. The preparations were connected to an isotonic transducer (load 0.5 g) in such a way as to record contractions mainly from the longitudinal axis and allowed to equilibrate for 60 min before the start of experiment: contractions were recorded using Bioscience transducers.

8.3. Contractility test

8.3.1. Spasmogenic assays

The crude extracts was prepared in stock solution of 20 mg/ml in DMSO and cumulatively added to the organ bath from concentration of 10, 25, 50, 100, 250, 500, 750 and 1000 µg/ml. The effective concentration of DMSO in the waterbath was less than 5%in all the experiments. Effect of the extracts on spontaneous motility of the ileal preparations were monitored at 20 min contact time for each concentration and cumulative dose-dependent curves for the extracts were determined to measure stimulatory effects.

8.3.2. Spasmolytic assays

8.3.2.1. Effects on acetylcholine-induced contraction

Acetylcholine hydrogen chloride was added to the organ bath cumulatively in the absence of test extracts at concentration ranging between 0.01-1.00 µg/ml in water. The process was repeated with addition of ACH (0.01-1.00 µg/ml) after 20 min pre-incubation of the isolated ileum with the extracts (10, 25, 50, 100, 250, 500, 750 and 1000 µg/ml).

8.3.2.2. Effects on Serotonin-induced contraction

Serotonin was added to the organ bath cumulatively in the absence of test extracts at concentration ranging between 0.001 - 0.1 µg/ml. The process was repeated with addition of 5-HT after 20 min pre-incubation of the isolated ileum with the extracts (10, 25, 50, 100, 250, 500, 750 and 1000 µg/ml).

8.3.2.3. Effects on K-induced contraction

The isolated ileum preparation was washed with K^+ free Kreb's solution (composition (g/l): NaCl, 6.94; KCl, 0.354; KH_2PO_4, 0.163; $NaHCO_3$, 2.1; $MgSO_4$, 0.370; $CaCl_2$, 0.367; glucose, 2.07 and pH 7.4) for 20 min after equilibration and incubated with the extracts for 20 min. Thereafter, KCl solution (100 µl) was added cumulatively.

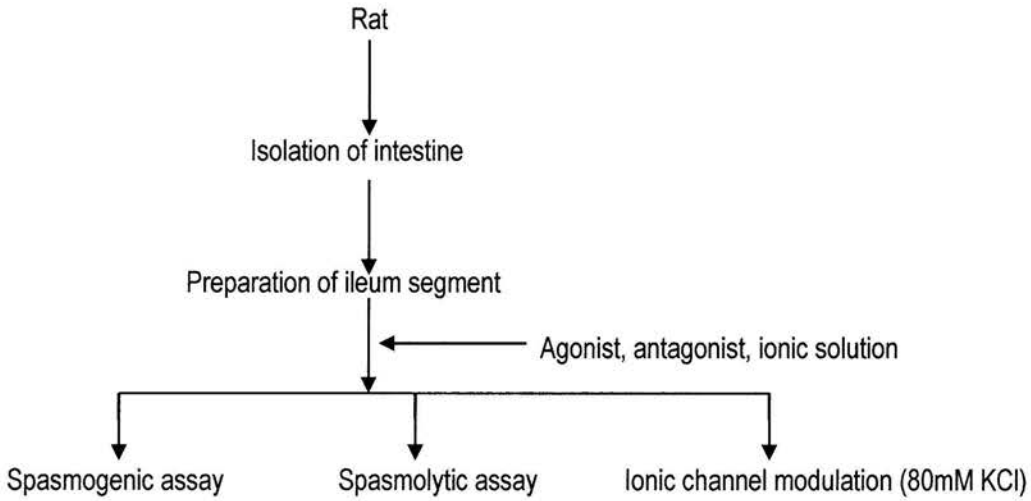


Fig. 8.1. Schematic presentation of the contractility assay

8.4 Data analysis

The inhibition of ileum contraction by test sample was normalized and expressed as a percentage of mean± SEM from 3-4 experiments of the references responses induced by acetylcholine (10 µg/ml), other spasmogens, receptor agonists and antagonists using the following formula:

$$\% \text{ Inhibition} = [A_C - A_T/A_C] \times 100$$

Where A_C is the amplitude (cm) of the ileum contraction induced by the agonists and antagonists in the absence of the test sample; A_T is the amplitude (cm) of the ileum contraction by the agonists and antagonists in the presence of the test sample. The changes in EC_{50} will be used to compare the effect of the extracts using an ANOVA.

8.5. Results.

8.5.1. Effect of *B. galpinii* crude extract on isolated rat ileum

The 70% acetone extract which should have high concentration of phenolics of *B. galpinii* (10 - 1000 µg/ml) stimulate spontaneous contraction of the rat ileum as shown in Fig 8.2 with EC_{50} value of 27.85 µg/ml. Maximum contraction (E_{max}) of 44 mm was obtained at 200 µg/ml and additional doses causes suboptimal response but

increase duration of response caused an irreversible spasm at the maximum dosage of 1000 µg/ml. Repeated administration of the extract at maximum dosage (1000 µg/ml) caused exhaustion of the ileum.

Effects of the extract on acetylcholine, serotonin, K^+ induced contractions and acetylcholine in the presence of atropine (acetylcholine non-specific muscarinic receptors antagonist) indicated dual mechanisms of being an agonist (prokinetic) and an antagonist (relaxant) agent. The extract also exhibited additive contractility activity with acetylcholine and agonistic tendency to serotonin-induced contraction of the isolated rat ileum (Fig 8.3 and 8.4).

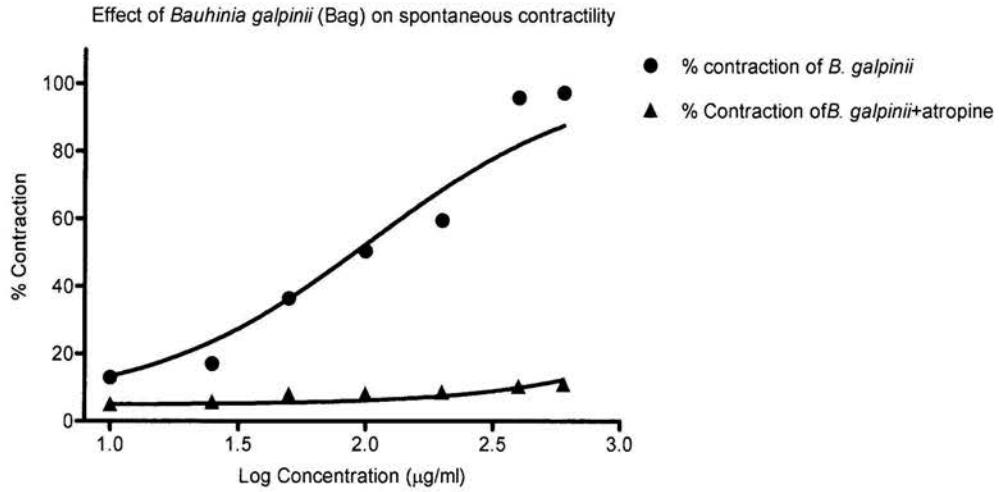


Fig.8.2: Stimulatory effect of 70% acetone leaf extract of *B. galpinii* on spontaneous contractility of isolated rat ileum and the antagonised effect of atropine.

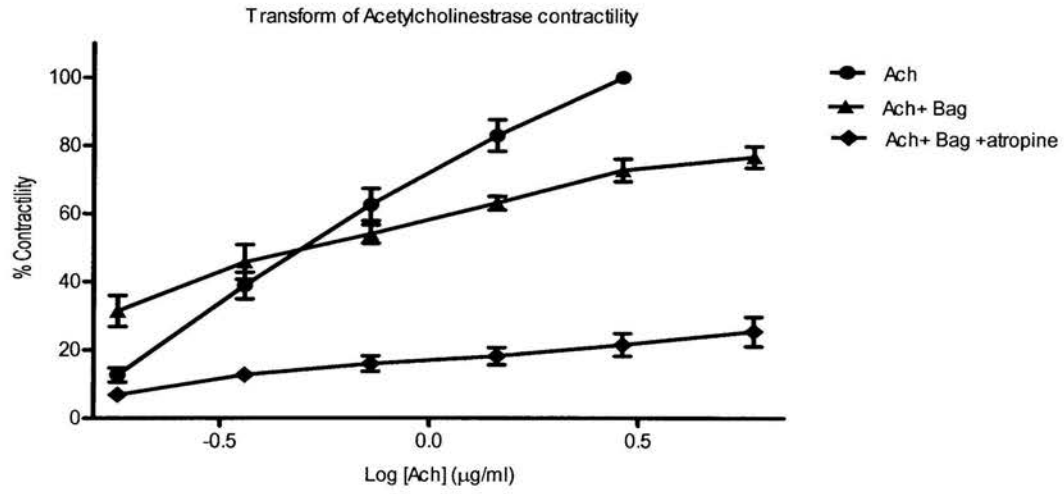


Fig. 8.3. Effect of 70% acetone leaf extract of *B. galpinii* (200 µg/ml) on the acetylcholine cumulative concentration-effect curves in the presence and absence of atropine

From the concentration-response curve (CRC) for acetylcholine-induced contraction, the EC_{50} value in the absence of *B. galpinii* was 0.033 µg/ml and the EC_{50} in the presence of *B. galpinii* was 0.049 µg/ml. The stimulation of spontaneous contraction and agonistic effects on acetylcholine-induced contraction were partially abolished by atropine (Fig 8.3). In the CRC for serotonin-induced contraction, the EC_{50} value in the absence of *B. galpinii* was 0.0025 µg/ml and the EC_{50} in the presence of *B. galpinii* was 0.0014 µg/ml. In contrast, the *B. galpinii* extract resulted in a concentration-dependent spasmolytic effect (antagonist) on K^+-induced contraction of the isolated rat ileum (Fig. 8.5) with maximum effect (E_{max}) of 40.66±5.13 mm at concentration of 200 µg/ml.

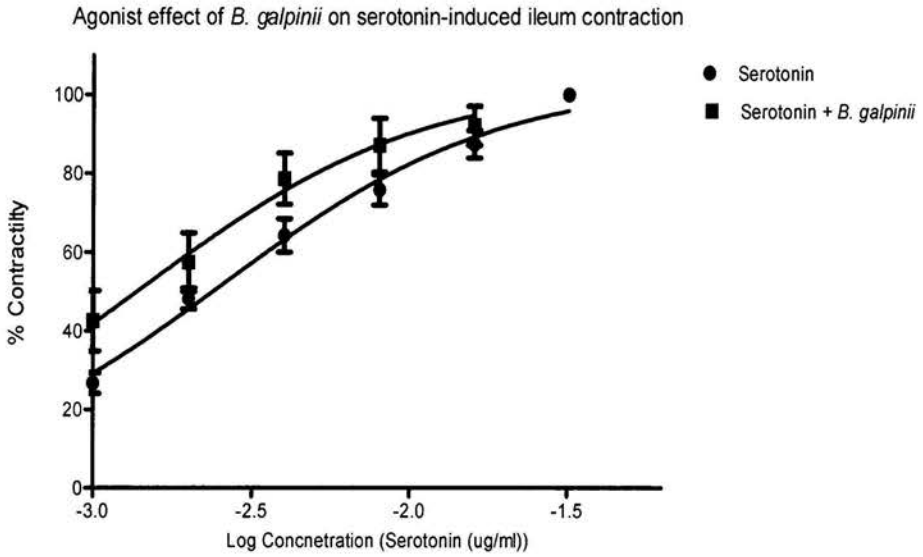


Fig. 8.4. Agonised effect of 70% acetone leaf extract of *B. galpinii* (200 µg/ml) on serotonin induced-contraction on rat isolated ileum.

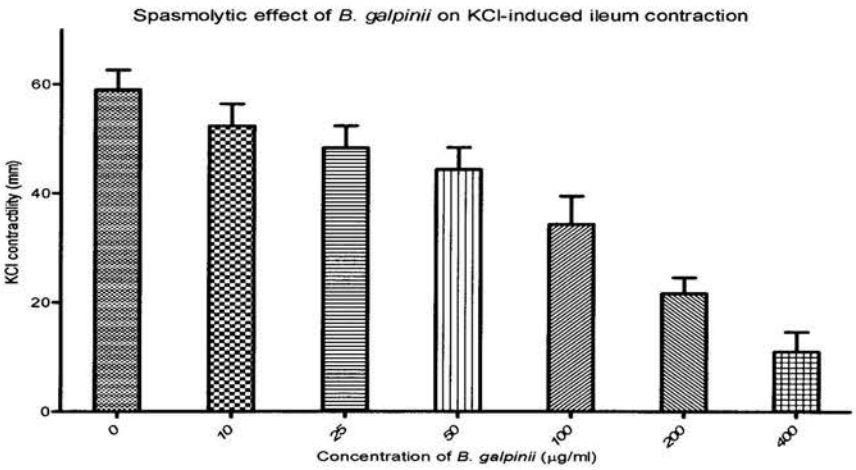


Fig. 8.5. Relaxant effect of 70% acetone leaf extract of *B. galpinii* on KCl induced contractility of isolated rat ileum

8.5.2. Effect of *C. vendae* crude extract on isolated rat ileum

The phenolic-enriched extract leaf extract of *C. vendae* do not stimulate spontaneous contraction (spasmogenic) of the isolated rat ileum, we therefore conclude that the extract have spasmolytic potential. The crude extract of *C. vendae* exhibited concentration-dependent spasmolytic effect on acetylcholine-induced contraction with EC_{50} values of 0.037, 0.027, 0.117, 0.365, and 0.396 µg/ml at the concentration of 0, 100, 200, 400, and 600 µg/ml of *C. vendae* in the organ bath (Fig 8.6) and concentration-dependent spasmolytic effect on serotonin-induced contraction of isolated rat ileum with EC_{50} value of 0.0017, 0.0044 and 0.012 µg/ml at the concentration of 0, 100, 200 µg/ml of *C. vendae* in the organ bath respectively (Fig 8.7). Equivalent volume of the solvent (5% DMSO) used in dissolving the extract had no effect on the spontaneous contraction or on 5-HT-induced contraction.

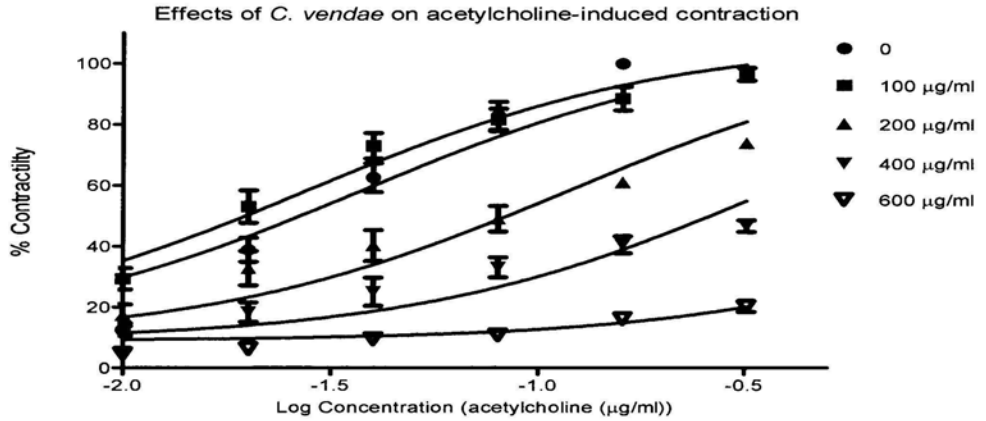


Fig.8.6. Spasmolytic effect of 70% acetone leaf extract of *C. vendae* on Ach-induced contractility of isolated rat ileum

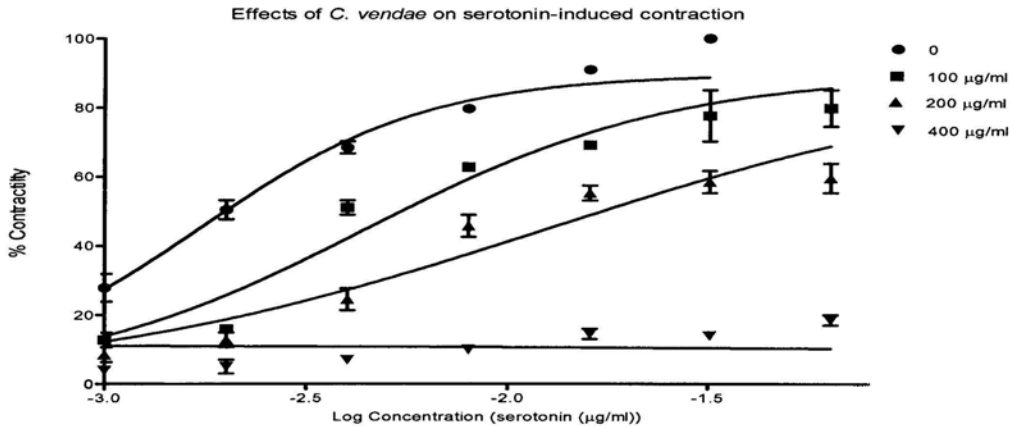


Fig.8.7. Relaxant effect of 70% acetone leaf extract of *C. vendae* on 5-HT-induced contractility of isolated rat ileum

Addition of depolarised KCl solution (80mM) caused sustained contractions which were inhibited by *C. vendae* phenolic enriched leaf extracts in concentration-dependent response (Fig. 8.8). Therefore, agent that inhibits contraction induced by depolarised KCl solution is considered to be a calcium channel blocker (Godfraind et al., 1986). The spasmolytic effects were reversible and the spontaneous contraction returned to normal after washing three times with kerbs' solution.

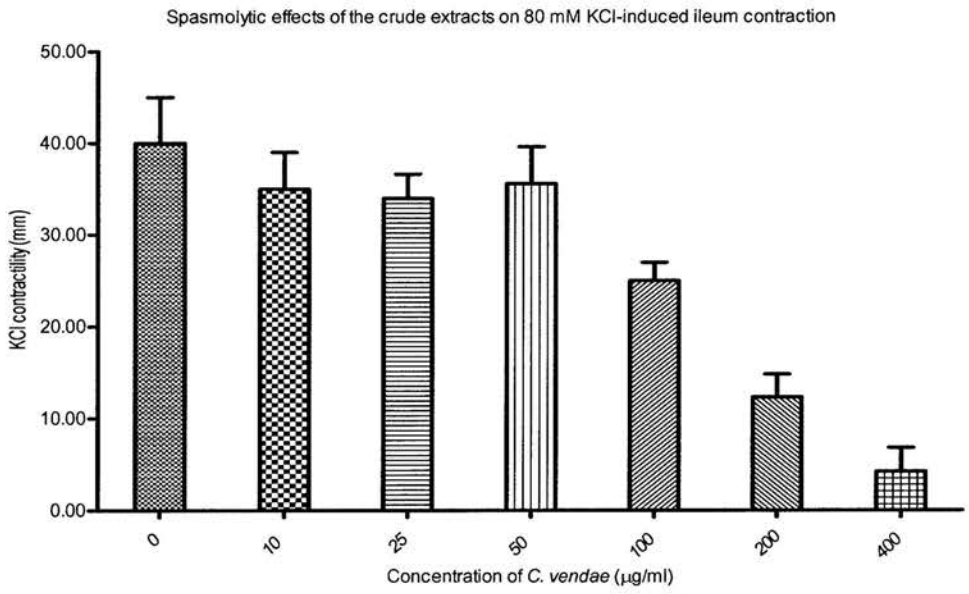


Fig. 8.8. Spasmolytic effect of the C. vendae on the depolarised KCl-induced isolated rat ileum contractions

8.6. Discussion

Gastrointestinal motor tone is modulated through multiple physiological mediators which include neurotransmitters, inflammatory mediators and oxidative metabolites (Hoogerwerf and Pasricha, 2006). The release of these chemical modulators in GIT causes stimulatory effect mediated through an ultimate increase in cytosolic Ca^{2+} (Burks, 1987). Drug substances with ability to block or alter any of the above pathways or with non-receptor specific inhibitory action such as Ca^{2+} antagonists could be considered to be effective as therapeutic agent in hyperactive or hypoactive GIT disorders. These are important in control or alleviating diseases such as diarrhoea, constipation, emesis and dyspepsia. To study the pharmacology and possible mechanism of smooth muscle excitatory or inhibitory effect of drugs and medicinal plant extracts isolated tissue preparations of laboratory animal are usually used for *in vitro* assays.

Acetylcholine (ACh) is a neurotransmitter released by the parasympathetic nervous system mediating its action in the GIT by stimulation of nicotinic acetylcholine receptors (nAChR) and muscarinic acetylcholine receptors (mAChR). In the GIT, five subtypes of the muscarinic receptors, namely M_1, M_2, M_3, M_4 and M_5 have been identified (Tobin *et al.* 2009). However, M_2 and M_3 receptors play some essential roles in the smooth muscle

contraction/relaxation of GIT (Matsui *et al.*, 2002; Takeuchi *et al.*, 2005; Unno *et al.*, 2005). Through this mechanism, acetylcholine plays a critical physiological role in regulating the peristaltic movements of the GIT (Brown and Taylor, 1996). The possible mechanisms responsible for contractility mediating action of drugs including medicinal plant extracts may include one or combinations of:

- Stimulation/inhibition of ACh release from the cholinergic nerve endings.
- Stimulation/inhibition of acetylcholinesterase (AChE) enzyme at the neuro-effector junction.
- Direct activation/inactivation of the muscarinic receptors of all smooth muscles, including those of GIT.

The effects of serotonin in the ENS are complex and diverse including modulation of smooth muscle function (promoting both contraction and relaxation), potent intestinal secretagogue (predominantly pro-secretory) and responses to visceral pain. Serotonin (5-HT) receptors found within the ENS and motor neurones of the GIT include 5-HT_1, 5-HT_2, 5-HT_3, 5-HT_4, 5-HT_5, 5-HT_6 and 5-HT_7. However, only 5-HT_1, 5-HT_2, 5-HT_3, 5-HT_4 and 5-HT_7 receptor subtypes are known to affect GIT motor functionality.

The 5-HT_3 and 5-HT_4 receptors are the most studied subtypes with the regards to physiological function and histological distribution in GIT (Chetty *et al.*, 2006; Cellek *et al.*, 2006). The 5-HT_3 receptor induces a rapid depolarization of the mesenteric neuron through enhancing ACh release (Kim, 2009), while 5-HT_4 receptor expressed in the nerve terminal facilitates the releases of neurotransmitters including ACh, substance P and vasoactive intestinal peptides (Kim, 2009; Wouters *et al.* 2007). These cellular events of 5-HT lead to an up-stream regulation enhancing the excitatory activity of GIT smooth muscles through mediating the ACh release. Serotonin is involved in cholera toxin-and bile salt-induced fluid and electrolyte secretion by activating the ENS.

Contractions of all smooth muscles, including those of GIT depend on the presence of Ca^{2+}. Increase and decrease in intracellular free Ca^{2+} are the principal mechanisms that initiate contraction and relaxation respectively in smooth muscle (Sanders, 2001). Agonists-induced contractions are related to the release of intracellular Ca^{2+} from sarcoplasmic stores and extracellular Ca^{2+} influx through L-type channels (Makhlouf, 1994). Therefore smooth muscle relaxations can be effected by antispasmodic drugs through the inhibition of Ca^{2+} entry or release into the cells. Exposure of smooth muscle cells to high concentration of K^+ (>30 mM) stimulate contractions through opening of voltage-dependent L-type Ca^{2+} channels and influx of extracellular Ca^{2+} (Bolton, 1979; Godfraind *et al.*, 1986).

The results obtained in this work indicated that phenolic-enriched crude leaf extract of *B. galpinii* contracted the rat ileum dose-dependently and its initial contractile phase was partially blocked by atropine, a naturally occurring alkaloid and well-known non-selective muscarinic receptor antagonists. Atropine competes with Ache and other muscarinic agonists for a common binding site on the muscarinic receptor. This result shows the involvement of

cholinergic muscarinic receptors alongside with other stimulatory receptors exhibiting initial contraction by *B. galpinii* on isolated rat ileum.

The phenolic-enriched crude leaf extract of *B. galpinii* also exhibited dose-dependent stimulating activity on serotonin-induced contraction of isolated rat ileum. The spasmogenic effects of the extract on ileum longitudinal muscle may be direct erotogenic activation of 5-HT receptor pathways or through the enhanced release of other neurotransmitters without erotogenic potential.

Addition of KCl (80 mm) caused sustained contractions which were inhibited by *B. galpinii*. Therefore the inhibitory effect of the crude extract of *B. galpinii* against K^+-induced contractions can be as result of the blockade of Ca^{2+} channels. Thus it can be concluded that *B. galpinii* has a dual-mechanism of action (prokinetic and relaxant) on gastro-intestinal motility, depending on the prevalent patho-physiological condition. The *B. galpinii* 70% acetone leaf extract can therefore be clinically relevant as therapeutic agent in diarrhoea and constipation which are both diseases with aetiology based on motility disturbances to a large extent.

Fumaria indica crude extract also has dual-spasmogenic and spasmolytic effects on isolated organs (Gilani *et al.*, 2005a). The aqueous-ethanolic extract (80% ethanol) of the aerial parts of *Hibiscus rosasinensis* Linn (Malvaceae) contains spasmogenic and spasmolytic constituents mediating their effect through cholinergic receptors activation and blockade of Ca^{2+} influx, respectively (Gilani *et al.*, 2005b). Crude aqueous leaf extracts of *Morinda morindoides* (Baker) Milne-Redh (Rubiaceae) agonise spontaneous contractility of isolated rat ileum (Cimanga *et al.*, 2010). The petroleum ether soluble fraction and the crude saponin constituents of the extract are responsible for the spasmogenic activities. The spasmogenic and spasmolytic effect of a particular medicinal plant extract on the isolated ileum depends on predominant phytochemical constituents. Phenolic compounds exhibit spasmolytic activity while saponins are responsible for the spasmogenic activities of many plant extract preparation. From the phytochemical analysis of the extract of *B. galpinii*, the extract contains high content of phenolics. However, the result obtained in this study indicated that the crude extract of *B. galpinii* also contains other active ingredients with spasmodic effect higher than the anticholinergic effect of the phenolics.

C. vendae extract did not stimulate spontaneous contractility of the rat ileum. Further investigation of its effects on ACh-induced contraction led to a concentration-dependent inhibitory activity against ACh contraction of the rat ileum. Anti-contractility effects of *C. vendae* against ACh-induced contraction are similar to atropine indicating that the extract may be acting via nAChR or mAChR.

Addition of KCl (80 mM) caused sustained contractions which were inhibited by both *B. galpinii* and *C. vendae* phenolic enriched leaf extracts in concentration-dependent response. Agents that inhibit contraction induced by KCl are considered to be a calcium channel blocker (Godfraind *et al.*, 1986). The spasmolytic effects were

reversible and the spontaneous contraction returned to normal after washing three times with Ca^{2+} free-Krebs solution.

The results indicate that *C. vendae* extract is capable of mediating spasmolytic effects on isolated rat ileum through multiple inhibitions of a wide range of contractile stimuli, such as neurotransmitters (acetylcholine and serotonin) and high potassium (depolarizing stimulus). This suggests that the ileum relaxant effects of the extract are not specific to a type of receptor but rather due to either general receptor inactivation or membrane depolarization. Muscarinic receptor antagonists, 5-HT receptor antagonist and Calcium channel blockers of the L-type are known to be effective as antispasmodic, anti-motility and antidiarrhoeal agents (Lee *et al.*, 1997; Brown and Taylor, 2006; Pasricha, 2006). Hence, the presence of multiple acting spasmolytic activities in the plant extract might be contributing towards its effectiveness in diarrhoea and abdominal spasm. The isolated triterpenoids such as ursolic acid, maslinic acid, corosolic acid, asiatic acid and arjunolic acid from the plant also have good antimicrobial activity and the stilbenoid glycosides such as combretastatin B5-O-2'-β-D-glucopyranoside and combretastatin B1-O-2'-β-D-glucopyranoside has good antioxidant activity. Such activities of the plant could account for additional benefits providing a wider cover for its use in diarrhoea of different aetiologies. This is also in accordance with the general understanding that plants contain multiple active constituents with effect enhancing activities (Gilani and Rahman, 2005).

8.7. Conclusion

The result indicated the *B. galpinii* have dual activities with the capacity of acting as prokinetic and spasmolytic agent while *C. vendae* acts as spasmolytes against the three spasmogens used to induce contraction of the ileum.Further studies aiming to identify the targeted receptor subtype and the type of interaction with muscarinic receptors as well as the identification of the main active principle are needed.

The results indicate that there is a scientific rationale for using extracts of these plant species to treat diarrhoea in humans or animals. In the next section some of the antimicrobial and anti-oxidant compounds present in these extracts will be isolated and characterized.

CHAPTER NINE

Isolation and characterization of antimicrobial and antioxidant compounds from *Bauhinia galpinii* and *Combretum vendae*

9.0. Introduction

One of the cardinal objectives in medicinal plant research and development is identification, isolation and characterization of the bioactive components present in an extract. Medicinal plant extracts are inherently complex mixture of diverse chemical components. Separation of the active components of plant phytochemicals from the inactive components are categorized into three parts: extraction, purification and chromatography. Extraction and purification involved sample preparations schematically represented in Fig. 9.1 and Fig. 9.2. Various chromatographic methods are available for qualitative and quantitative (TLC fingerprint, high performance liquid chromatography (HPLC) fingerprints) as well as for isolations (open column chromatography (OCC), vacuum liquid chromatography (VLC), HPLC, high-speed counter-current chromatography (HSCCC), gas-liquid chromatography (GLC) and/or gel permeation chromatography (GPC)). The principles of separation are based on molecular size, adsorption to the stationary phase, polarity and solubility in the mobile phase.

Structural information on isolated compounds are usually obtained from different spectroscopic techniques namely: nuclear magnetic resonance spectroscopy (NMR), mass spectroscopy (MS), and to a lesser extent infrared spectroscopy (IR) and ultraviolet-visible spectroscopy (UV-visible).The characteristic features of each NMR experiments are summarized in Table 9.1.

Table 9.1 NMR experiments commonly applied for natural product structural elucidation (Simpson *et al.*, 2011)

NMR experiment	**Information/interpretation**
Proton NMR (1D ^{1}H NMR)	Quantitative overview of the distribution of protons in a sample.
Carbon-13 NMR (1D ^{13}C NMR)	Can provide a quantitative overview of the carbon distribution.
Distortionless enhancement through polarization transfer (DEPT) (1D ^{13}C NMR)	Separate the carbon of a compound into primary (CH_3), secondary (CH_2), Tertiary (CH) and quaternary (C) spectra.
^{1}H-^{1}H Correlation spectroscopy (COSY) (2D ^{1}H NMR)	Connectivity information of protons on adjacent carbons. Cross-peaks connect the chemical shifts of protons that are coupled. Symmetrical cross peaks appear around a central diagonal.
^{1}H-^{13}C Heteronuclear single quantum correlation (HSQC) and heteronuclear multiple quantum correlation (HMQC) (2D ^{1}H- ^{13}C NMR)	^{1}H–^{13}C 1 bond correlation. Cross peaks represent carbon chemical shifts in one dimension and proton chemical shifts in the other dimension.
^{1}H-^{13}C Heteronuclear bond multiple correlation (HMBC) (2D ^{1}H- ^{13}C NMR)	^{1}H–^{13}C 2–4 bond correlations. Quaternary carbons are observed. Connectivity information is read as vertical lines.

9.1.1. Column chromatography

Isolation of such compounds is usually carried out by open column chromatography under gravitation force using silica gel, sephadex, polyamides or reverse phase (RP) mode on C8 or C18-bonded silica gel stationary phase. The separation of individual compounds from the complex extract mixture is based on the compound characteristic ability for the stationary phase in the column relative to the polarity of the mobile phase. Changing the polarity (gradient elution) of the mobile phase will allowed all target compounds to elute in a sequential manner. The chromatographic process should be rapid, do not lead to decomposition of compounds, material loss, or formation of artefacts.

Open column chromatography is simple, cheap and universally practiced despite some obvious disadvantage of method being slow and often produces irreversible adsorption of sample onto the stationary phase. The method is also encumbered with large sample and solvent requirement. The bioactive compounds of interests in this project are non-volatile.

9.1.2. Mass spectrometry

Mass spectrometry (MS) is an important physico-chemical tool applied for structural elucidation of compounds from natural products including medicinal plants. The fundamental principle of MS is the use of different physical means for sample ionization and separation of the ions generated based on their mass (m) to charge (z) ratio (m/z) (Rijke *et al.*, 2006). The ionization techniques available include electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI), electron ionization (EI), chemical ionization (CI), fast atom bombardment (FAB), and matrix-assisted laser desorption ionization (MALDI) (Rijke *et al.*, 2006). Mass spectrometry has high sensitivity with detection limit of fentogram compared to NMR with sensitivity limit of nanogram range and above (Simpson *et al.*, 2011). The high sensitivity and the flexibility for hyphenation with other chromatographic technique made MS a versatile analytical instrument.

9.2. Materials and Methods

9.2.1. Preparation of plant extracts

The extraction and fractionation protocol was followed as described in Chapter 3. The schematic diagrams of the extraction, fractionation and isolation processes for *Combretum vendae* and *Bauhinia galpinii* are presented in Fig 9.1 and 9.2.

9.2.2. Bioautography

The bioautography against bacteria (*E. coli and S. aureus*) protocol were followed as described in chapter 4 while the TLC-DPPH antioxidant assay were carried out as described in chapter 5.

9.2.3. Isolation of bioactive terpenoids from *Combretum vendae*

The n-Hexane and ethyl acetate fractions showed one and two clear zone(s) of microbial growth inhibition respectively. The two fractions were subjected to gravitational column chromatography on silica gel (2.5cm×73cm using 150 g silica, particle size 0.063–0.200 nm, Merck 70–230 mesh ASTM) using the solvent mixture of hexane: ethyl acetate starting with 100% n-Hexane, 99: 1, 98:2, 97:3, 96:4, 95:5, 94:6, 93:7 and finally 90:10 as mobile phase. Schematic representation of the isolation procedure is presented in Fig 9.1.

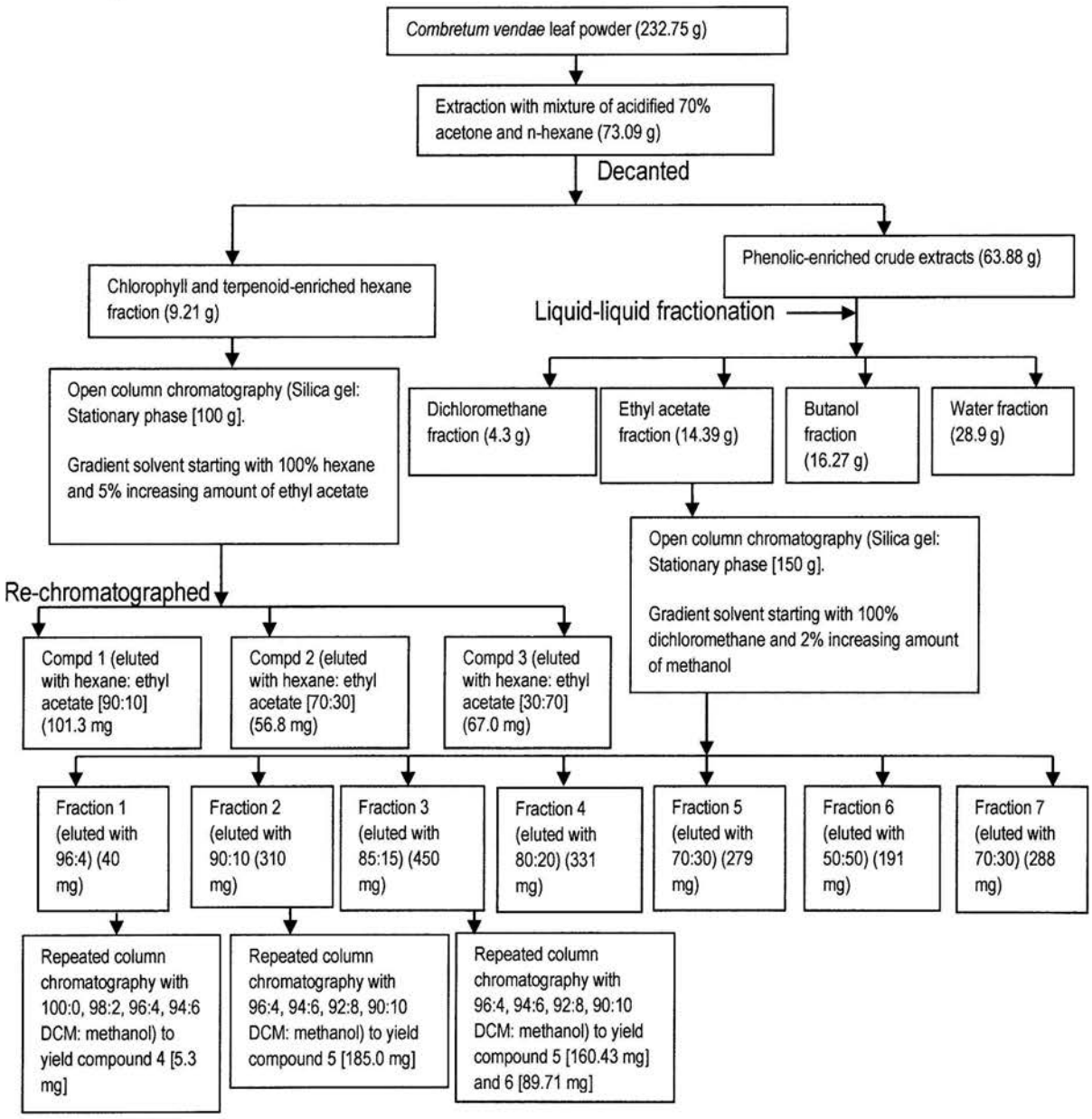


Fig. 9.1. Extraction, fractionation and isolation of bioactive compounds from the leaf extract of *Combretum vendae*

The dried fractions were reconstituted (10 mg/ml) and 10 µl of the aliquot spotted on TLC. Three mixtures of n-Hexane: ethyl acetate (90:10; 95:5 and 98:2) was used to develop the plates. The fractions with R_f corresponding to the R_f values of bioautography assay were combined. The purification of the compounds was achieved by repeated column chromatography until single spot was obtained for each compound using three different mobile phases to develop the TLC.

9.2 4. Isolation of bioactive phenolics from *Combretum vendae*

The ethyl acetate, n-Butanol and residual water fractions exhibited good antimicrobial and antioxidant activities. In TLC-DPPH assay four clear zones of antioxidant components were observed in ethyl acetate fraction. The fraction was subjected to open column chromatography under gravitational force (2.5cm×73cm using 150 g silica, particle size 0.063–0.200 nm, Merck 70–230 mesh ASTM) using the solvent mixture of dichloromethane: methanol starting with 100% dichloromethane, 98:2, 96:4, 94:6, 92:8, 90:10, 85:15, 80:20, 70:30, 60:40, 50:50 as mobile phase. The eluents were monitored with DPPH-TLC antioxidant assay and vanillin/H_2SO_4 spray reagent. The fractions with the same compounds were combined and subjected to further cleaning by re-chromatography until single spots were obtained on TLC chromatogram using three different mobile phases. Schematic representation of the isolation procedure is presented in Fig 9.1.

9.3. Isolation of compounds from *B. galpinii*

9.3.1. Isolation of bioactive terpenoid from *Bauhinia galpinii*

The n-Hexane fractions showed one clear zone of microbial growth inhibition and was subjected to open column chromatography under gravity on silica gel (2.5cm×73cm using 150 g silica, particle size 0.063–0.200 nm, Merck 70–230 mesh ASTM) using the solvent mixture of hexane: ethyl acetate starting with 100% n-Hexane, 98:2, 96:4, 94:6, 92:8 and finally 90:10 as mobile phase. The eluent were monitored using TLC and vanillin/H_2SO_4 spray. The fractions containing the target compound was combined and the chromatography process was repeated until a single spot using three mixtures of n-Hexane: ethyl acetate (90:10; 95:5 and 98:2) as mobile phases for TLC chromatogram was obtained. Schematic representation of the isolation procedure is presented in Fig 9.2.

9.3.2. Isolation of bioactive phenolics from *Bauhinia galpinii*

The ethyl acetate, n-Butanol and residual water fractions exhibited good antimicrobial and antioxidant activities. In TLC-DPPH assay four clear zones of antioxidant components were observed in ethyl acetate and butanol fractions. The fractions were subjected to open column chromatography under gravitational force (2.5cm×73cm using 150 g silica, particle size 0.063–0.200 nm, Merck 70–230 mesh ASTM) using the acidified solvent mixture of dichloromethane: methanol starting with 100% dichloromethane, 98:2, 96:4, 94:6, 92:8, 90:10, 85:15, 80:20,

70:30, 60:40, 50:50 as mobile phase. The eluents were monitored with DPPH-TLC antioxidant assay and vanillin/H_2SO_4 spray reagent. The fractions with the same compounds were combined and subjected to further cleaning by re-chromatography using silica gel or Sephadex L20 until single spots were obtained on TLC chromatogram using three different mobile phases. Schematic representation of the isolation procedure is presented in Fig 9.2.

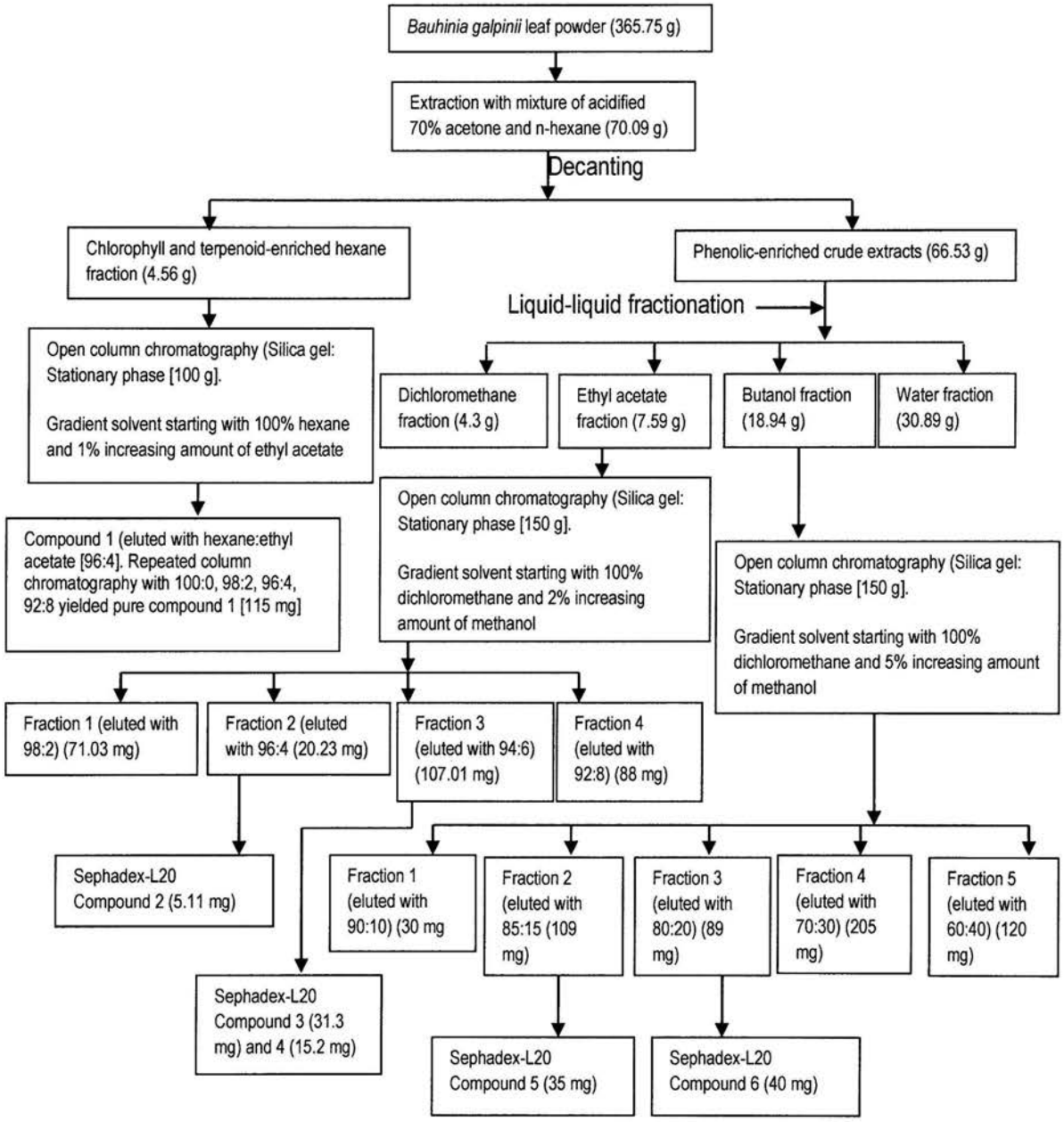


Fig. 9.2. Extraction, fractionation and isolation of bioactive compounds from the leaf extract of *Bauhinia galpinii*

9.4 Characterization of the isolated compounds

9.4.1 NMR spectroscopy

One dimensional (1D) (^{1}H and ^{13}C) and two dimensional (2D) NMR spectra (^{1}H-^{1}H COSY, HMQC and HMBC) NMR spectra were recorded on a Varian-NMR-vnmrs 600 spectrometer with tetramethylsilane (TSM) as internal standard. Standard pulse sequences were used for homo- and heteronuclear correlation experiments. ^{1}H NMR spectra were measured at 599 MHz whereas ^{13}C NMR spectra were run at 150 MHz. Multiplicities of ^{13}C NMR resonances were determined by DEPT experiments. All NMR experiments were performed at constant temperature (27 °C) using software supplied by the manufacturer, employing deuteriochloroform, deuteriomethanol, or deuteriodimethylsulphoxide as solvent on the basis of solubility of the sample and literature data.

9.4.2 Mass Spectroscopy:

Electrospray ionization mass spectrometric analyses (negative and positive mode) were carried out to obtain the molecular weight and fragmentations patterns of the isolated compound(s) using TOF mass spectrometer (WATERS HPLC).

9.4.3 UV spectroscopy:

The UV-spectrum of the isolated bioactive compound(s) was recorded using Agilent 1200 UV-Visible spectrophotometer.

9.5 Results

9.5.1 Identification of the chemical structures of isolated compounds from *Combretum vendae*

The n-Hexane and ethyl acetate fractions from the acidified 70% acetone leaf extract of *Combretum vendae* A. E. van Wyk through bioassay guided fractionation were repeatedly subjected to gravity column chromatography to yield one pure, mixture of four position isomer antimicrobial triterpenoids and two stilbene glucopyranoside. The structures of the compounds were determined by extensive NMR techniques and chemical methods mainly by 1D NMR (^{1}H, ^{13}C and DEPT) and 2D NMR (HSQC, HMBC and COSY), ESIMS, UV-visible spectra and by comparison with the literature data. The chemical structures of the compounds compounds isolated from *Combretum vendae* are presented in Fig 9.3.

Compound 1 was obtained as an amorphous white powder. Detailed analyses of the 1D and 2D NMR spectra indicated the presence of 30 carbons which revealed 7 methyl, 9 methylene, 6 methane, 6 quaternary, 1 carboxylic acid at δ_C 178.9, an olefinic broad triplet proton at δ_H 5.25 (H-12) coupled to a carbon at δ_C 125.4, a quaternary carbon at δ 138.8.0 (C-13) and a β-18 proton at δ_H 2.05 characteristic of signal of an ursol-12-en

skeleton (Appendix 9.1). The Comparison of these NMR data with the literature confirms the compound as ursol-12-en-28-oic acid (ursolic acid) (Mahato and Kundu, 1994).

Compound 2 and 3 were obtained as position isomeric mixture; the TLC fingerprint indicated unresolved single spot with three different mobile phases. However, the 1D and 2D NMR spectra exhibited chemical shift characteristic of both olean-12-ene and ursol-12-ene. ^{13}C NMR (DMSO): δ 179.7(C-28, olean), 178.0 (C-28, urs), 145.08 (C-13, olean), 122.72 (C-12, olean), 139.42 (C-13, urs), 125.55 (C-12, urs), 41 (C-18, olean), 53 (C-18, urs), 27.97 (C-19, olean), 39.07 (C-19, urs) and 46.13 (C-20, olean), 39.02 (C-20, urs) (Appendix 9.2). Comparing the data with the literature values, the two compounds in the mixture were identified to be corosolic acid and maslinic acid (Mahato and Kundu, 1994)

Compound 4 and 5 were also obtained as position isomeric mixture. The spectra 1D and 2D were similar to the spectra of compound 2 and 3 except the presence of additional hydroxyl at C-24) (appendix 9.3). From the correlation data and literature values, compound 4 and 5 were identified to be asiaitic acid and arjunolic acid respectively (Mahato and Kundu, 1994).

Compound 6 was obtained as an amorphous powder from the ethyl acetate fraction. The proton NMR spectrum shows signal for apigenin: δ_H 6.19 (d, J=2.0 Hz, H-6), 6.38 (d, J=2.0 Hz, H-8), 7.2 (d, J=2.1 Hz, H-3), 6.50 (d, J=8.9 Hz, H-3' and H-5'), and 7.30 (d, J=2.1 Hz, 8.9, 2H' and H-6'). The ^1H NMR spectral data correspond to the literature values (Zhang *et al.*, 2011).

Compound 7 was obtained as creamy glass-like solid mass and deduced to have molecular formula $C_{23}H_{29}O_{11}$ with molecular weight 481 by EIMS. The ^{13}C-NMR spectrum indicated 17 carbon signals due to aglycone and six carbon signals of a glycoside group. The signal consist twelve aromatic signals, three methoxy and two aliphatic (Appendix 5). The ^1H-NMR spectra revealed the presence of two aromatic benzylic rings. The ^1H detected heteronuclear multiple-bond connectivity (HMBC) spectrum indicated long-range correlations from ^1H-1a'(δ_H 2.9, 3.0) to C-1' (δ_C 128.55), C-2' (δ_C 144.35), C-6' (δ_C 118.95), C-1a (δ_C 36.96) and C-1 (δ_C 132.72). Long range correlation observed between ^1H-1a (δ_H 2.7) and C-1 (δ_C 132.72, C-2 (δ_C 106.16), C-6 (δ_C 106.16), C-1a' (δ_C 31.73) and C-1' (δ_C 128.55). Additional long-range correlation were observed between ^1H-2, 6 (δ_H 6.5) and C-1 (δ_C 132.72), C-3, C-5 (δ_C 148.16), C-4 (δ_C 133.79), C-1a (δ_C 36.96); ^1H-5' (δ_H 6.7) to C-1' (δ_C 128.55), C-3' (δ_C 139.71), C-6' (δ_C 118.95), C-4' (δ_C 147.26) and ^1H-6' (δ_H 6.6) to C-1a' (δ_C 31.73), C-3' (δ_C 139.71), C-4' (δ_C 147.26). Long-range correlation also observed between methoxyl signal (δ_H 3.7) and the C-3, C-5 (δ_C 148.16), C-4' (δ_C 147.26) (appendix 9.4). Based on these data, the aglycone of compound 7 was determined to be stilbenes derivative. The signals for anomeric proton and carbon (δ_H 4.5 and δ_C 106.26) indicated the presence of a sugar moiet. The long-range correlation in the HMBC experiment between the anomeric proton signal (δ_H 4.5) of the β-D-glucopyranosyl group and the C-2' signal (δ_C 144.35) confirmed the position of the attachment of the

glucopyranosyl moiety on the phenolic ring. The structure of compound 6 was determined to be combretastatin B5-O-2'-β-D-glucopyranoside. The EIMS, 1D (^{1}H and ^{13}C, DEPT), and 2D (HSQC, HMBC, COSY) data correlate with literature information on the compound (Pettit *et al.*, 1985).

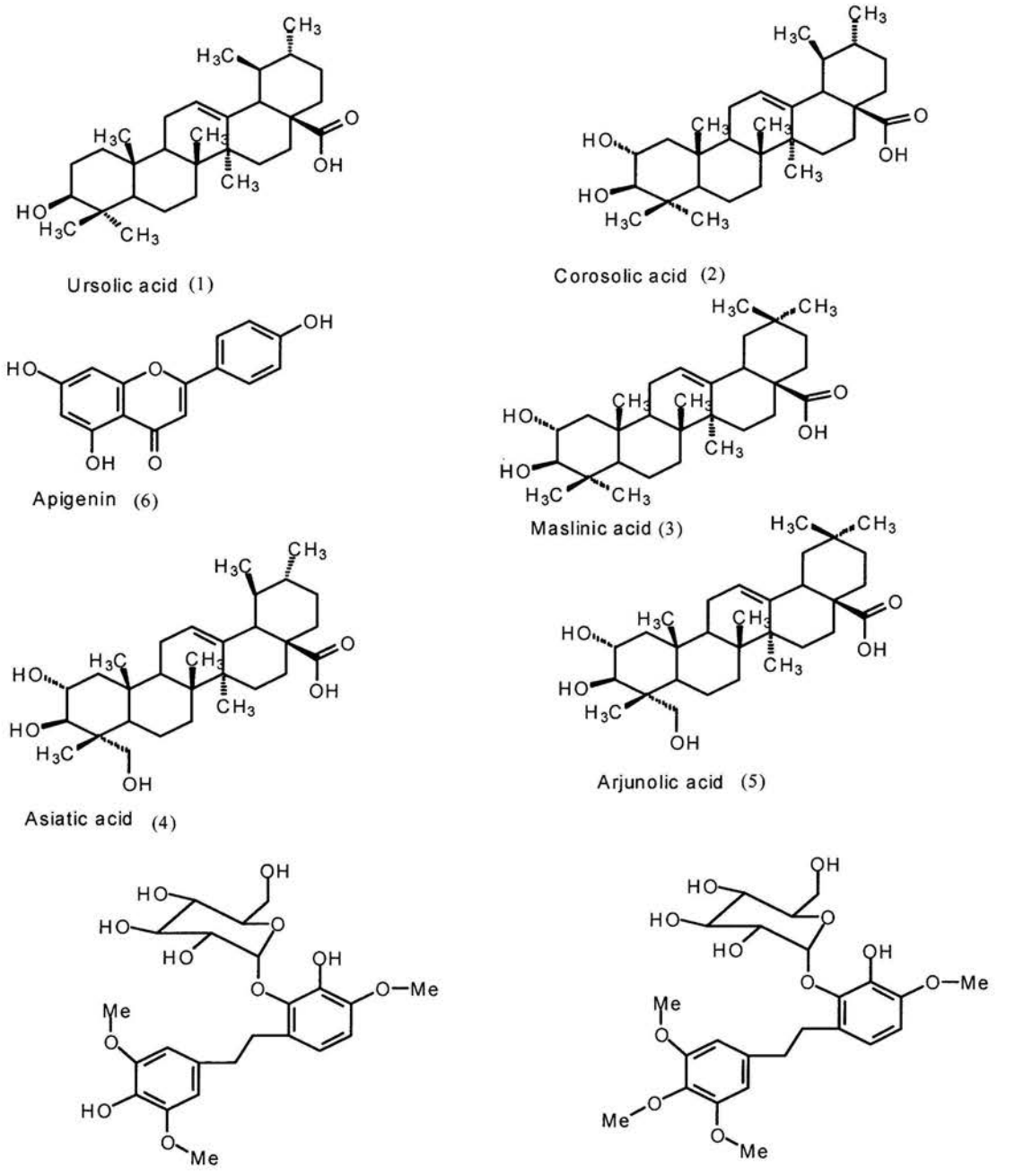


Fig 9.3 Chemical structures of isolated bioactive compounds from 70% acetone leaf extract of *Combretum vendae*

Compound 8 had molecular weight of 496 and the molecular formula was deduced to be $C_{24}H_{32}O_{11}$ by EIMS. The compound differs from compound 6 by the presence of extra methyl group. This was confirmed by 1D and 2D NMR spectra. From HSQC one additional methoxyl signal (δ_H 3.6 and δ_C 59.69) having long range correlation C-4 (δ_C 135) (appendix 9.5). The long range correlation from HMBC experiment between ^{1}H-4 proton of the glucopyranosyl moiety (δ_H 3.7) and the C-2' signal (δ_C 143.78) confirmed the position of the structure of compound 7 to be combretastatin B1-O-2'-β-D-glucopyranoside. The EIMS, 1D (^{1}H and ^{13}C, DEPT), and 2D (HSQC, HMBC, COSY) data correlate with literature information on the compound (Schwikkard *et al.*, 2000).

9.5.2 Antimicrobial assay of isolated compound from *C. vendae*

Antimicrobial activities of the isolated compounds against standard and clinical isolate pathogens expressed as MIC are presented in Table 9.1. Some of the compounds exhibited good microbial growth inhibitory potential worthy of pharmacological considerations with MIC ranging from 3.9-31 µg/ml.

Table 9.2: Minimum inhibitory concentration (µg/ml) of the isolated compounds from the leaf extract of *Combretum vendae*

Microorganisms	Compound 1	Compounds 2 and 3	Compounds 4 and 5	Compound 7	Compound 8
E. coli	62	250	250	250	250
E. faecalis	31	31	31	31	31
S. aureus	62	125	125	125	62
P. aeruginosa	125	125	62	250	250
C. albicans (M0825)	15	7.8	7.8	31	31
C. albicans (M0824)	62	62	31	125	125
C. albicans (1051604)	3.9	3.9	3.9	3.9	3.9
C. albicans (1051608)	7.8	7.8	7.8	15	15
C. albicans (ATCC 10231)	7.8	15	7.8	31	31
C. neoformans	15	15	15	125	125
A. fumigatus	31	31	31	250	250

1: Ursolic acid, 2 and 3: mixture of maslinic and corosolic acid, 4 and 5: mixture of asiatic and arjulonic acid, 6: combretastatin B5-O-2'-β-D-glucopyranoside.7: combretastatin B1-O-2'-β-D-glucopyranoside. *C. albicans* (M0824), *C. albicans* (M0825), *C. albicans* (1051604), *C. albicans* (1051608) are clinical isolate obtained from National Health Laboratory Service, Pretotia, South Africa.

9.5.3 Identification of the isolated bioactive compounds from *Bauhinia galpinii*

The combine TLC fingerprint, TLC-DPPH assay and bioactivity guided fractionation of acidified 70% acetone leaf extract of *B. galpinii* detected four major flavonoids from ethyl acetate fraction with antioxidant activity. Bioautography against fungal and bacterial pathogens revealed two microbial inhibitory growth spot in Hexane

fraction and one in DCM fraction respectively. The bioactive compounds were isolated from each fraction using open column chromatography with silica gel as stationary phase. The phenolics compounds were further purified using Sephadex L-20 as stationary phase and acetone/methanol (50:50) as mobile phase at a rate of 2ml/5min. The chemical structures of the compounds were determined by detailed nuclear magnetic resonance (NMR) techniques including the one dimensional (1D) NMR (proton (^{1}H), carbon-13 (^{13}C) and distortion enhancement DEPT) and two dimensional (2D) NMR (HSQC, HMBC and COSY). Mass spectrometry and the fragmentation patterns of the compounds were extensively used for the structural elucidation.

Compound 9 obtained as white amorphous powder from hexane fraction was characterized by ^{1}H NMR spectra (in $CDCl_3$), ^{13}C-NMR spectra (in $CDCl_3$), HSQC, HMBC, DEPT and COSY. The ^{1}H NMR spectrum of 9 showed a one-proton doublet at δ_H 5.33 (J¼5.5 Hz) assigned to a vinylic H-6 proton. A one-proton broad multiplet at δ_H 3.46 with half-width of 18.5 Hz was attributed to carbinol H-3 proton. Two three-proton broad signals at δ_H 0.67 and 1.01 were attributed to a tertiary C-18 and C-19 methyl protons. A six-proton broad signal at δ_H 0.83 was associated with C-29 and C-20 methyl protons. Two three proton doublets at δ_H 0.91 (J¼6.2 Hz) and 0.85 (J¼6.3 Hz) were due to secondary C-26 and C-27 methyl protons. The remaining methylene and methine protons appeared between δ_H 2.50 and 1.27. The presence of all the methyl signals in the range δ_H 1.01–0.67 suggested that all these functionalities were located on the saturated carbons. The ^{13}C NMR spectrum of 9 exhibited signals for vinylic carbons at δ_C 140.97 (C-5) and 121.96 (C-6). The two carbinol signals appeared at δ_C 72.04 (C-3) and 76.86 (CH_3 $\underline{CH_2}O$-) respectively. The carbon signals in the upfield region at δ_C 12.20, 21.30, 19.25, 20.04, 19.62, 12.08 and 19.00 were associated with the methyl functionalities. The remaining methylene and methine carbon resonated between δ_C 56.99 and 23.29. The ^{1}H–^{1}H COSY spectrum of 9 showed correlations of H-6 with H2-7 and H-8; H-3 with H2-2, H2-4 (appendix 9.6). The ^{1}H and ^{13}C NMR spectral data of steroidal nucleus of 9 were compared with related steroidal constituents (Alam *et al.*, 1994). On the basis of the foregoing discussion, the structure of 9 was elucidated as 3β-ethyl stigmast-5-en-ol (3β-ethyl sitosterol). This compound may be an artefact of sitosterol.

Compound 10 and 11 was isolated from the ethyl acetate fraction as a yellow powder by repeated gravitational column chromatography on silica gel and Sephadex L-20 stationary phases. The ^{1}H NMR spectrum in deuterated methanol of 10 showed signals for quercetin: δ_H 6.19 (d, J=2.0 Hz, H-6), 6.38 (d, J=2.0 Hz, H-8), 7.57 (d, J=2.1 Hz, H-2', δ_C 116.30), 6.86 (d, J=8.9 Hz, H-5', δ_C 116.78), and 7.56 (d, J=2.1 Hz, 8.9, H-6', δ_C 121.65). Carbon 13 NMR (100 MHz, in ppm, methanol-d4) shows 15 signals and the data (appendix 6) correlated well with literature for 3, 5, 7, 3', 4'-pentahydroxyflavone (Quercetin) (Said *et al.*, 2009)

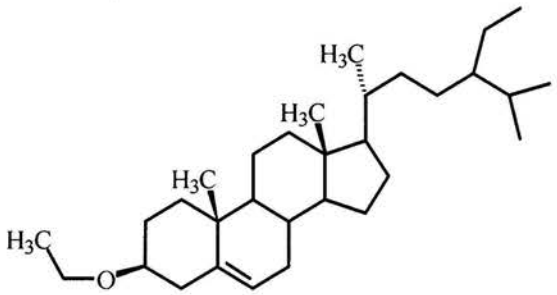
The ^{1}H NMR of compound 11 in deuterated methanol at 599.74MHz revealed the presence of myricetin aglycone: δ_H 7.383 (s, H-2',6', δ_C 107.09) for the B phenyl ring, and 6.369 (d, H-8, δ_C 92.95), 6.338 (d, H-8, δ_C 92.95), 6.172 (d, H-6, δ_C 97.80) and 6.170 (d, H-6, δ_C 97.80) for the meta substituted A phenyl ring. The structure

was confirmed by ^{13}C-NMR which showed 15 signals without methoxy or glycoside substituent and 2D correlation (HSQC and HMBC) (appendix 9.7). The data correlated well with literature data for 3, 5, 7, 3', 4', 5'-hexahydroxyflavone (myricetin) (Said *et al.*, 2009)

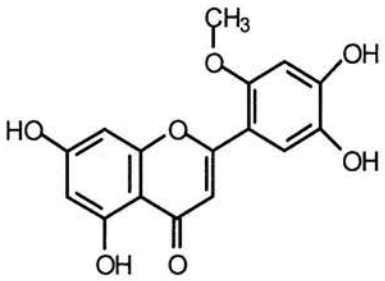
The NMR spectrum of compound 12 was typical for a flavone with one meta-substituted and para-substituted phenolic moiety. The UV spectrum showed λ_{max} 261.27 and 369.27 nm which compared favourably with 263 and 367 nm reported by Abdurrahman and Moon, 2007 for isoetin moiety. The compound was assigned molecular formula $C_{16}H_{10}O_7$ with the aid of a peak observed in the ESI-MS experiment at 315.054. The carbon chemical shifts were assigned by the combination^{1}H-^{13}C HSQC and long range couplings in the ^{1}H-^{13}C HMBC experiments. The ^{1}H-NMR spectrum was indicative of five aromatic protons, one methoxyl group. The ^{1}H and ^{13}C NMR spectra data correlated with those of isoetin flavone moiety (Abdurrahman and Moon, 2007; Pauli and Junior, 1995; Voirin *et al.*, 1975) (Table 1). The ^{1}H-^{13}C HSQC correlations were used to assign signal at δ_H 7.19, 6.27 and 6.40 to the protons at C-3 (s, δ_C 108.85), C-6 (d, J = δ_C 99.97) and C-8 (d, J = δ_C 94.86) positions of A ring, and the signal at δ_H 6.65 and 7.38 to the proton at C-3' (s, δ_C 101.36) and C-6' (s, δ_C 114.45) positions of B ring of the isoetin moiety respectively. The long range coupling in the HMBC presented in appendix 9.10 also supported the isoetin flavone moiety. The methoxy protons signal at δ_H 3.8 was correlated with the quaternary carbons (C-2') at δ_C 153.28 indicating the attachment of methoxy group at the carbon (appendix 9.8). From all the correlations, compound 13 was determined to be a new flavone named as 5, 7, 4' 5' tetrahydroxy-2'-methoxyflavone (isoetin 2'-methyl ether) or 5, 7, 2' 5' tetrahydroxy-4'-methoxyflavone (isoetin 4'-methyl ether)

Compound 13 and 14 obtained as yellow powder respectively. UV spectra in MeOH showed λ_{max} of 203.27, 255.27, 355.27 nm for compound 14, and 207.27, 257, 354.27 nm for compound 13 respectively. The ESI–MS peaks of compound 13 and compound 14 in negative mode, were observed at m/z 463 [M-H] - and m/z 479 [M-H] - respectively. The molecular ion of compound13 was 16 mass units smaller than that of compound14, which corresponds to the difference in the number of hydroxyl groups on the B-ring of the flavonol aglycone. The ^{1}H NMR spectrum in deuterated methanol of 13 showed signals for quercetin: δ_H 6.19 (d, J=2.0 Hz, H-6, δ_C 99.17), 6.38 (d, J=2.0 Hz, H-8, δ_C 94.09), 7.57 (d, J=2.1 Hz, H-2', δ_C), 6.86 (d, J=8.9 Hz, H-5', δ_C), and 7.56 (d, J=2.1 Hz, 8.9, H-6', δ_C) and anomeric protons at δ_H 5.192 (s, δ_C 104.14) and 5.22 (s, δ_C 104.14) characteristic of galactopyranose. The ^{1}H NMR of 14 in deuterated methanol at 599.74 MHz revealed the presence of myricetin aglycone with five aromatic proton: δ_H 6.19 (s, H-6, δ_C 98.48), 6.38 (s, H-8, δ_C 93.25), 7.37 (s, H-2', H-6', δ_C 108.52) and similar anomeric protons to those in 13 (appendix 9.9). The ^{13}C NMR and HMBC, HSQC spectra in deuterated methanol at 150 MHz of 13 and 14 were very similar, except for the signals corresponding to the flavonol aglycone (appendix 9.8). The spectral data and correlation with literature information revealed the two compounds to be quercetin-3-O-β-galactopyranoside (Rayyan *et al.*, 2005; Yan *et al.*, 2002) and myricetin-3-O-β-

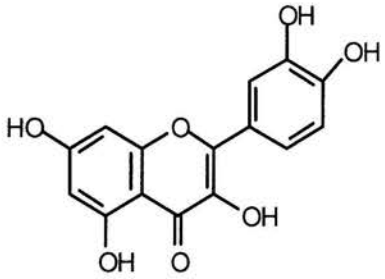
galactopyranoside (Yan *et al.*, 2002) respectively. The chemical structures of the isolated compounds from *Bauhinia galpinii* are presented in Fig. 9.4.



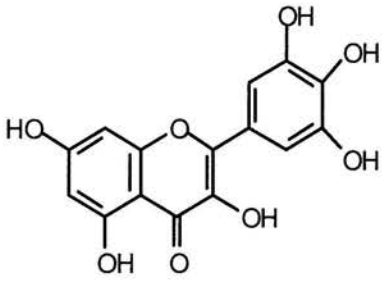
3-beta ethyl sitosterol (9)



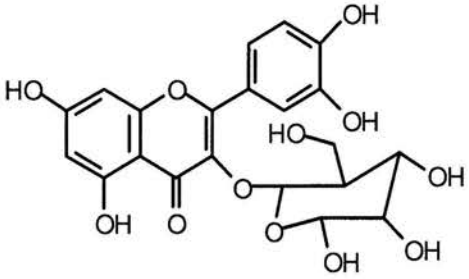
5, 7, 4', 5' tetrahydroxy-2-methoxyflavone
(Isoetin 2'-methyl ether) (10)



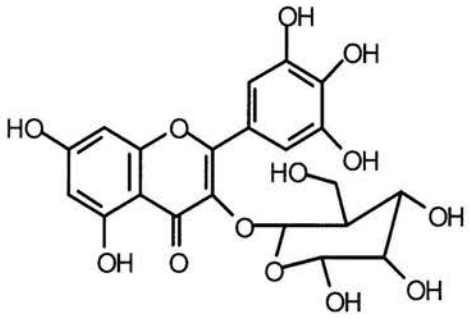
3, 5, 7, 3' 4' Pentahydroxyflavone
(Quercetin) (11)



3, 5, 7, 3' 4', 5' Hexahydroxyflavone
(Myricetin) (12)



Quercetin-3-O-beta galactopyranose (13)



Myricetin-3-O-beta galactopyranose (14)

Fig. 9.4. Chemical structure of bioactive compound isolated from the leaf extract of *Bauhinia galpinii*

9.5.4. Antimicrobial assay of isolated compounds from *B. galpinii*

Antimicrobial potential of the isolated compounds against some diarrhoeal pathogens and organisms of other important infectious diseases are presented in Table 9.2 as minimum inhibitory concentration (MIC) (µg/ml).

Table 9.3. Minimum inhibitory concentration (µg/ml) of the isolated compounds from the leaf extract of *Bauhinia galpinii* and positive control

Microorganisms	9	10	11	13	14	Gentamicin	Amphoteric B
E. coli	125	31	31	31	62	15.5	-
E. faecalis	31	31	7.8	62	62	7.75	-
S. aureus	62	62	15	31	31	3.87	-
P. aeruginosa	125	62	15	15	15	3.87	-
C. albicans (M0825)	15	3.9	3.9	31	31	-	3.9
C. albicans (M0824)	31	31	31	31	31	-	3.9
C. albicans (1051604)	3.9	3.9	3.9	3.9	3.9	-	0.8
C. albicans (1051608)	62	3.9	3.9	15	15	-	1.93
C. albicans (ATCC 10231)	62	3.9	3.9	31	31	-	1.93
C. neoformans	125	31	31	125	125	-	7.75
A. fumigatus	125	31	31	125	125	-	7.75

1: 3β-ethyl sitosterol; 2 Quercetin; 3 Myricetin, 4 Quercetin-3-O-β-galactopyranose; 5 Myricetin- 3-O-β-galactopyranose. *C. albicans* (M0824), *C. albicans* (M0825), *C. albicans* (1051604), *C. albicans* (1051608) are clinical isolate obtained from National Health Laboratory Service, Pretotia, South Africa.

9.6. Discussion

9.6.1. Bioactive compounds from *Combretum vendae*

Bioactivity guided investigation of *Combretum vendae* afforded ursolic acid ((3β)-3-hydroxyurs-12-en-28-oic acid), mixture of asiatic and arjunolic acid, mixture of maslinic and corosolic acid, apigenin, Combretastatin B5-2'-O-β-glucopyranoside and Combretastatin B1-2'-O-β-glucopyranoside all exhibiting broad based microbial growth inhibitory potentials.

Ursolic acid and its derivatives biosynthetically derived from the cyclization of squalene (and other triterpene acids) have been extensively studied as pharmacological active molecules in many *in vitro* and *in vivo* studies. Some of the biological activities include antioxidant, hepatoprotective, anti-inflammatory, anticancer, anti-HIV, vasorelaxant (Aguirre-Crespo *et al.*, 2006) and antidiabetic activities. Several mechanisms have been proposed to explain its anti-inflammatory activity, including inhibition of secretory PLA_2 enzymes, IL-1β secretion, iNOS and COX-2. Ursolic acid have no antispasmolytic effect (Estrada-Soto *et al.*, 2007), however the broad base antimicrobial activities (3.9-125 µg/ml) obtained in this work and other reports of antioxidant, anti-inflammatory and antidiabetic provide pharmacological bases for further investigation of the compound as antidiarrhoeal agent.

Biological activities such as α-glucosidase inhibition of arjunolic acid (18.63±0.32 µg/ml), asiatic acid (30.03±0.41 µg/ml), maslinic acid (5.52±0.19 µg/ml) and corosolic acid (3.53±0.27 µg/ml) isolated from *Lagerstroemia speciosa* were reported. The α-glucosidase inhibition of these compounds shows their antidiabetic and antiadhesion potential against microbial pathogens both of which are also important in antidiarrhoea therapy. However the compounds have no α-amylase inhibitory activities (Hou *et al*., 2009). Mixtures of arjunolic acid and asiatic acid isolated from Combretum nicholsonii have antifungal activities with MIC of 0.2-1.5 µg/ml. However, antimicrobial activities obtained in the work is slightly higher for the mixture (3.9-250 µg/ml), the difference in result may be due to experimental variable such as concentrations of the culture media, composition of the mixture, incubation time and strains. These results indicate that individual compound or the mixtures have pharmacological potentiality against infectious pathogens.

Apigenin have been isolated previously from the acetone leaf extract of *C. vendae*. The antibacterial activity of the compound was evaluated (Eloff *et al*., 2008).

Combretastatin B5-2'-O-β-glucopyranoside have been isolated previously from the seed of *Combretum kraussii*. The aglycone moiety of this compound was isolated from the acetone leaf extract of *Combretum woodii* reported to have antibacterial activity. This is the first report on the isolation of the compound from the leaf extract and its antimicrobial activities.

Combretastatin B1-2'-O-β-glucopyranoside have been previously isolated from the seeds of *Combretum kraussii* (Pettit *et al*, 1987) and wood bark of *Combretum erythrophylum* (Schwikkard *et al*., 2000). The stilbenes have been reported to have cytotoxic activity with effect on tubulin polymerization, the primary protein component of microtubules in cancer hence the potential of the compound as anticancer drug is being explored. The compound has been evaluated for selective inhibitory activity against the DNA-damaging repair-deficient strain of *Saccharomyces cerevisiae* deficient in the RAD52 recombination repair gene and exhibited no activity while the derivative with unsaturated bond at 1aC- 1a'C (combretastatin A1-2'-O-β-glucopyranoside) was active (Schwikkard *et al*., 2000). This indicated the importance of the unsaturated bond in the structure-activity relationship for cytotoxicity effects. However, there is no literature report on other biological activity potential such as antimicrobial, antioxidant, anti-inflammatory and antidiarrhoea. This is the first report on the isolation of the compound from the leaf extract and its antimicrobial activities. The non-cytotoxic effect of the compound against the cancer cells lines ((Schwikkard *et al*., 2000) indicates that the compound can be exploited for other biological activities.

9.6.2. Bioactive compounds from *Bauhinia galpinii*

Isoetin (5, 7, 2' 4' 5'-pentahydroxyl flavone) and its various derivatives are rare compounds formed by insertion of a 2' hydroxyl group into luteolin to give characteristic yellow pigments in some plant part Lattanzio *et al.*, 2006). 5, 7, 4' 5' tetrahydroxy-2'-methoxyflavone (isoetin 2'-methyl ether) is a new compound (unfortunately the quantity obtained was not enough for bioassays). A related compound 5, 7, 2' 4' tetrahydroxy-5'-methoxyflavone (isoetin 5'-methyl ether) isolate from *Trihosanthes kirilowii* (Cucurbitaceae) was reported to be cytotoxic against human lung cell line A549 (IC_{50} 0.92 µg/ml), human melanoma Sk-Mel-2 (IC_{50} 8.0 µg/ml), and mouse melanoma B16F1 cell lines (IC_{50} 7.23 µg/ml). High cytotoxicity (IC_{50} 2.5 µg/ml) of the acetone root extract of *B. galpinii* against Vero cell lines has been reported (Samie *et al.*, 2009). Isolation of more 5, 7, 4' 5' tetrahydroxy-2'-methoxyflavone (isoetin 2'-methyl ether) from *B. galpinii* for further studies on cytotoxicity effect important.

Quercetin and myricetin are the common flavonol present in dietary and constitute the active component of medicinal plant with characteristic hydroxyl substitutions at the 3, 5, 7, 3', 4' (Quercetin), and 3, 5, 7, 3', 4' and 5' (myricetin) positions of flavone ring. The compounds occur in nature mostly as glycoside with D-glucose, L-rhamnose, D-galactose or arabinose rather than free aglycone. Biological effects of phenolic compounds depend on their bioavailability which is determined by the lipophilicity of each molecule. The mechanisms involved in digestion and absorption of phenolic compound is complex but passive transports through the membrane have been proposed. The glycosides and methylated phenolics are not readily absorbed in native form but need to be hydrolysed by intestinal enzymes or colonic microflora to aglycone before absorption.

Quercetin and myricetin alongside their galactopyranoside derivatives were isolated from the acidified 70% acetone leaf extract of *Bauhinia galpinii.* Quercetin galactopyranose and myricetin galactopyranose were previously isolated from this plant and their antioxidant activity and cytotoxicity was evaluated (Aderogba *et al.*, 2007).

A wide range of biological activities related to diarrhoeal pathogenesis including antimicrobial (Naz *et al.*, 2007), anti-inflammatory and spasmolytic due to their antioxidant and/or free radical scavenging (Aderogba *et al.*, 2007) as well as ability to interfere with several enzymatic pathways have been reported for the compounds. Quercetin and myricetin are active against microorganisms of the genera *Bacillus*, *Corynebacterium*, *Salmonella*, *Shigella*, *Staphylococcus*, *Streptococcus*, and against *Escherichia coli* and *Vibrio cholerae* (Naz *et al.*, 2007).

Both compounds also have protective effects on intestinal TJ barrier function through interaction with intracellular signaling molecules, tyrosine kinases and protein kinase C δ (PKCδ) (Suzuki and Hara, 2010). The intercellular TJs are the major determinant of the intestinal physical barrier regulating the paracellular movement of ions, solute, and water through the intestinal epithelium. Impaired intestinal TJs functions are involved in several

intestinal and metabolic diseases, such as diarrhoea, inflammatory bowel disease and food allergy (Suzuki and Hara, 2010). Myricetin inhibits the generation of MDA a cytotoxic by-product of lipid peroxidation of arachidonic acid liberated from membrane phospholipids (Robak *et al*., 1986).

Myricetin has potential as an antiviral agent by its ability to inhibit the reverse transcriptase from Moloney murine leukaemia virus, Rauscher murine leukaemia virus and human immunodeficiency virus (Ono *et al*., 1990). Myricetin also has antidiabetic activity with ability to stimulate lipogenesis and enhanced glucose uptake into adipocytes. The mechanisms postulated include changes in lipid-protein interaction or increase membrane fluidity. Myricetin inhibits the intraluminal accumulation of fluid and prevent diarrhoea induced by castor oil.

9.7. Conclusion

All the isolated compounds from the two plant species have biological activities with relevance against one or more diarrhoeal pathophysiology. The antimicrobial of some of the compounds are worthy of pharmacological consideration. In order to exploit the full potentials of these compounds some *in vitro* and *in vivo* studies are required to determine the mechanism of action.

CHAPTER TEN

General conclusion and future prospects

10. Introduction

Diarrhoea is one of the major health challenges facing the world and especially developing countries. The problem is aggravated due to the increasing number of immunocompromised people infected by HIV, with associated opportunistic infections and other health complications manifesting as diarrhoeal symptoms. The emergence of more virulent strains resulting from drug resistant pathogens and the apparent side effects of some conventional drugs currently in use is also serious concerns in diarrhoeal control and management. In animal production, diarrhoeal outbreaks usually cause serious economic losses due to reduced productivity, cost of treatment, lower level of reproduction and increased mortality.

However, the success of oral rehydration therapy in reducing mortality and lack of commercial interest in drugs for developing countries has slowed the progress in the development of novel agents for treating diarrhoeal diseases. Therefore, there is an urgent need for new therapeutic drugs or herbal products with lower cost, high efficacy and little or no side effects. Plants and plant preparations have been used ethnopharmacologically in treating diarrhoea successfully, although their efficacies, mechanisms of action and safety have generally not been proven scientifically. Thus, the overall aim of this project was to systematically determine the efficacy, mode of action and safety of some plants used traditionally in South African traditional medicine as diarrhoea therapy. The following objectives were identified to attain this aim:

- To conduct comprehensive literature works on diarrhoeal aetiologies and mechanisms and, medicinal plants use for treating diarrhoea symptoms in Southern Africa.
- To determine the phenolic compositions of the crude extract.
- To evaluate the effects of some selected medicinal plant species against pathogenic microbes known to induce diarrhoea.
- To determine the antioxidative properties of the selected plants using various standard protocols.
- To determine the anti-inflammatory potentials of the selected plants using various standard protocols.
- To evaluate the toxicity risk of the crude extracts.
- To determine the intestinal motility modulatory effects of the most promising extracts on the contractility process of the isolated rat ileum induced by spasmogens and ion channels activators.
- To isolate and characterize the component(s) that exhibit antimicrobial and antioxidant properties from the most promising extracts.

The achieved objectives of the study are outlined as follows:

10.1. Identification of diarrhoeal pathogenesis and medicinal plants used as therapeutic agents

The data generated from the literature work indicate that diarrhoea has a number of pathogenesis such as microbial infection, chronic inflammation, oxidative injury to intestinal mucosal lining, and deranged intestinal motility. The mechanisms involved include one or combination of ionic and water secretion into the lumen and reduced absorption of fluid from the intestine. The compendium of the medicinal plants used as antidiarrhoeal agents (254 species) in Southern Africa also revealed the diarrhoeal challenges and the wide acceptability of medicinal for cure.

10.2. Antimicrobial evaluation of the extracts against infectious pathogens

The results obtained by the antimicrobial screening indicate the presence of many compounds with potent antibacterial and antifungal activity against the standard strains of microbes responsible for infectious diarrhoea and other important infectious diseases in humans and animals. The significant inhibitory activity exhibited by the water fraction of the *C. vendae* against *S. aureus* can be considered as important for the traditional use of this plant, where water is the main extractant available. Generally, the results revealed that the antimicrobial potential of the extracts are potentiated in the hexane and dichloromethane fractions. Future investigation of the potent extracts and fractions against resistant and virulent pathogens might indicate new mechanisms for the growth inhibition of the microorganisms.

10.3. Antioxidant evaluation of the extracts

The crude extracts and the polar fractions of ethyl acetate and butanol had significant radical scavenging activity, through hydrogen and proton donation mechanisms. These activities are ascribed to the presence of large quantity of phenolics. In view of the oxidative stress in the pathogenesis of diarrhoea through tissue injury by ROS/RNS involvement in lipid peroxidation, exacerbation of inflammatory processes and some of the reactive species serving as secretagogues, the strong antioxidant activity could indicate the presence of compounds with potentially important mechanisms of pharmacological relevance in reducing the deleterious effects of the oxidative species in diarrhoea. *In vitro* results however, cannot be literally translated into *in vivo* situation due the problem of bioavailability, absorption and possible metabolic transformations of the bioactive compounds in the intestine. Further research are needed to verify using other models with different mechanisms against substrates which are generated in human or animal cells as well as *in vivo* studies to evaluate their efficacy and safety.

10.4. Anti-inflammatory potential of the extracts

Inflammation is regarded as the hallmark of many diseases aetiology and the significant mediators are eicosanoids (prostaglandins, prostacyclin) from cyclooxygenase (COX) and leukotrienes from lipoxygenase (LOX) pathways. These two enzymes are the target for modulating the inflammatory process. The result obtained indicated that the polar extracts of *Bauhinia*, *Carissa*, and *Syzygium* species used for COX inhibitory assay were active against COX-1 with no activity against COX-2 while the *Combretum* species were inactive against both enzymes. COX-1 selective inhibitors are considered to cause GIT injury while selective COX-2 inhibitors are more beneficial against inflammatory processes, therefore these plant polar extracts should be used with caution because possible intestinal injury. Most of the plant extracts however, had good LOX inhibitory activity. Current research on anti-inflammatory agent focus on dual activity as COX and LOX inhibitors since both pathways uses the same substrate. If one pathway is closed down, more substrate will be available to the other unperturbed pathway, thus increasing its products and consequently promoting some other inflammatory mechanisms. Additional work is required to determine the fraction(s) in which the active component is present and the probable mechanisms of action. Since the polar extract are not active against COX-2, the non-polar extracts or fractions still have to be tested. The plant extracts also have to be tested against other inflammatory biomarkers and mediators including an *in vivo* studies using laboratory animal model.

10.5. Toxicity risk of the extracts

The toxicity risk assessment using MTT assay (Mosmann, 1983) using Vero African green monkey kidney cell lines indicated that the extracts of *Combretum* species except *C. bracteosum* were highly toxic. The other extracts have varying degree of toxicity with *Ozoroa mucronata* being the least toxic. 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 except perhaps cancer. The toxicity of medicinal plants depends on many factors such as the plant part used, and the solvents used as extractant which are determinant of the compositional characteristics and biological activity of extracts. Most of the highly toxic extracts also contain a high quantity of hydrolysable tannin. Poor handling of raw or processed materials may produce exogenous toxic contaminants not inherent as plant phytochemicals. It should be kept in mind that the results of cytotoxicity testing may vary considerably depending on the cell type used, the initial cell density to which the extracts are exposed, and the duration of exposure. Vero cells were selected as these are readily available and are commonly used in cytotoxicity tests. In this study, a low cell density was used and the cells were exposed for a long time, 5 days (McGaw et al., 2007). Hence, relatively low LC_{50} values were obtained and differences in cytotoxicity between the extracts were maximised. Further work is needed to test the extracts, fractions and subfractions against other cell lines and, also to conduct acute and chronic toxicity assays with a view of determining the toxic constituents present in the plants.

It is a pity that one of the two plant species selected for in depth work *C. vendae* had a high cellular toxicity. In future studies toxicity of extracts should be investigated at an early stage.

10.6 Motility modulatory effects of *Bauhinia galpinii* and *Combretum vendae*

Considering the wide ethnopharmacological use of *B. galpinii* and *C. vendae* against GIT disorders and their excellent activity in some of the preliminary screening, the two plants were chosen for motility modulatory assays despite the toxicity potential of *C. vendae*. This was with the view that the toxic component(s) will be determined and separated from the other active components. The data generated by the study indicate that *B. galpinii* has a dual-mechanism of action (prokinetic and relaxant) on gastro-intestinal motility while *C. vendae* extracts exhibited spasmolytic (relaxant) effects on isolated rat ileum through multiple mechanisms. These results were important as they indicate that *B. galpinii* extract can clinically be relevant as therapeutic agent in diarrhoea and constipation which are both diseases with aetiology based on motility disturbances to a large extent while the presence of multiple acting spasmolytic activities in the *C. vendae* extract might be contributing towards its effectiveness in diarrhoea and abdominal spasm therapy. Further work are needed for the identification of the specific ENS receptors through which these extract acts as well as the phytochemical compounds responsible for their activities.

10.7. Isolation and characterisation of antimicrobial compounds

Bioassay-guided protocols for antibacterial and antioxidant activity were adopted for the identification and isolation of 14 compounds (8 from *C. vendae* and 6 from *B. galpinii*) using open column chromatography with silica gel and Sephadex LH 20 as stationary phases. However, some of the compounds are mixtures of position isomers which are extremely difficult to separate. The compounds were characterised as ursol-12-en-28-oic acid (ursolic acid), a mixture of corosolic acid and maslinic acid, and a mixture of asiatic acid and arjunolic acid, two stilbenoid glycosides (combretastatin B5-O-2'-β-D-glucopyranoside and combretastatin B1-O-2'-β-D-glucopyranoside) and one flavone (apigenin) from the *Combretum vendae*.

One phytosterol (β-3 ethyl sitosterol), one new flavone (5, 7, 4' 5' tetrahydroxy-2'-methoxyflavone (isoetin 2'-methyl ether) or 5, 7, 2' 5' tetrahydroxy-4'-methoxyflavone (isoetin 4'-methyl ether)), two known flavonols (3, 5, 7, 3', 4'-pentahydroxyflavone (Quercetin) and 3, 5, 7, 3', 4', 5'-hexahydroxyflavone (myricetin)) and their galactoside derivatives (quercetin-3-O-β-galactopyranoside and myricetin-3-O-β-galactopyranoside) were isolated from *Bauhinia galpinii*.

The results from this study indicate that medicinal plants used in ethnopharmacology are reservoirs of bioactive compounds. Some of the medicinal plants may serve as potential sources of novel active compounds or lead molecule for synthesis of more potent drugs. There is also a distinct possibility of developing plants extracts that

could be used by poor rural people or sophisticated herbal medicines from some of the species investigated in this study. The information gained from this work provides a baseline study for other scientist to explore other medicinal plant species in depth with possible commercial application.

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Appendix 9.0: ETHNOBOTANICAL AND LITERATURE INFORMATION OF MEDICINAL PLANT SPECIES USED TRADITIONALLY FOR TREATING DIARRHOEA IN SOUTH AFRICA

Family/Plant species	Local names	Part used	Ethnopharmacological information	Biological activities investigated	Bioactive compound(s) isolated
Aizoaceae					
Carpobrotus acinaciformis (L.) L. Bolus	perdevy	Leaf juice	Sore throat, dysentery, mouth infection (van Wyk, 2008)	Antibactertial (Oskay *et al.*, 2009)	2-descarboxy-betanidin (Dembitsky, 2005)
Carpobrotus edulis (L.) L. Bolus	Ikhambi-lamabulawo. Umgongozi	leaves	Diarrhoea, digestive problems, allergy(Thring and Weitz, 2006); dysentery (van Wyk, 2008)	Antibacterial (Van der Watt *et al.*, 2001)	Rutin, hyperoside, neohesperidin, catechin, ferulic acid (van der Watt *et al.*, 2001)
Carpobrotus muirii (L.) L. Bolus		Leaves	Dysentery, digestive problem, mouth ulcers, thrush (Thring and Weitz, 2006)	Antimicrobial (Springfield *et al*, 2003)	-
Alliaceae					
Agapanthus praecox Willd.	uMkhondo (X)	Roots	Diarrhoea in sheep and goat (Dold and Cocks, 2001, McGaw and Eloff., 2008)	-	-
Tulbaghia alliacea L.f.	Umwelela X, ivimba-mpunzi X, Sikwa Z	bulb	Stomach ache, fever, tuberculosis, influenza (Bisi-Johnson *et al.*, 2010)	Antimycobacterial (Bamuamba *et al.*, 2008), Mutagenicty and antimutagenicity (Reid *et al.*, 2006), anticandidiasis (Thamburan *et al.*, 2006)	-
Amaranthaceae					
Guilleminea densa Moq	Sephatho (S)	Root	Decoction for diarrhoea (Mathabe *et al.*, 2006)	-	-
Hermbstaedtia odorata Wild	Ubuphuphu (X, Z)	leaves	Food and infusion for diarrhoea (Bisi-Johnson *et al.*, 2010); Root cleansing stomach wash alone or with *Acaccia xanthophloea* and (Hutchings *et al.*, 1996).	-	-
Amaryllidacceae					
Scadoxus puniceus (L.) Friis and Nordal	Umphompho-wezinja, Isiphompho umgola Z	Bulb and root	Stomach ache, diarrhoea, nausea (Bisi-Johnson *et al.*, 2010)	Antimicrobial, anti-inflammatory, acetylcholinesterase inhibition and mutagenic activities (Ndhlala *et al.*, 2010)	-
Anacardiaceae					
Mangifera indica L.	Umango	Leaves, bark	Diarrhoea (de Wet *et al.*, 2010)	Antidiarrhoeal (Sairam *et al.*, 2003), antidiabetic (Aderibigbe *et al.*, 2001	Gallotannins (Engels *et al.*, 2010), mangiferrin (Singh *et al.*, 2009).
Ozoroa insignis Delile	Monoko	Stem bark	Decoction for diarrhoea (Mathabe *et al.*, 2006), vinearal diseases, parasites, kidney	Antibacterial (Mathabe *et al.*, 2006); antigardial (Johns *et al.*, 1995),	6-pentadecylsalicylic acid (antifouling), tirucallane triterpenes (Liu and Abreu, 2006)

			trouble (Liu and Abreu, 2006)	antimalarial (Asase *et al.*, 2005), Cytotoxicity (Rea *et al.*, 2003), antischistosomiasis (Molgaard *et al.*, 2001; Ndamba *et al.*, 1994).	
Ozoroa mucronata (Bernh.ex C.Krauss) R.fern & A. Fern		root	Diarrhoea, intestinal parasites and stomach trouble (Yamagiwa *et al.*, 1987)	LOX inhibition, PG synthase inhibition (Kubo *et al*, 1987)	Anarcardic acid (LOX inhibition) (Ha and Kubo, 2005), Moronic acid (Hotesttmann Kaldas and Nakanishi, 1979)
Ozoroa paniculosa (Sond.) R. & A. Fernandes	Mubandulakhali, Mudumbula (V)	Bark, root bark	Abdominal problems in animal (Hutching *et al.*, 1996), Diarrhoea, sweating sickness (Van der Merwe *et al.*, 2001; McGaw *et al.*, 2008)	Antioxidant (Motlhanka, 2008), antimicrobial and antimycobacterial	-
Ozoroa schaerocarpa R. Fern & A. Fern	Mudumbula (V)	Bark	Infusion for diarrhoea (Sibandze *et al.*, 2010)	Antiescherichial (Sibandze *et al.*, 2010)	-
Protorhus longifolia (Bernh.ex C. Krauss) Engl.	i(u)Zntlwa, ikubalo, umkupati X	Bark	Heartwater and diarrhoea in cows (Dold and Cocks, 2001, McGaw and Eloff, 2008); Heart burn and stomach bleeding (Hutchings *et al.*, 1996)	Antimicrobial (Suleiman *et al.*, 2010)	-
Sclerocarya birrea (A. Rich.) Hochst. subsp. *caffra* (Sond.)	Mufula (V)	Leaves, bark, roots	Diarrhoea and fractures (Van der Merwe *et al.*, 2001; McGaw *et al.*, 2008)	Mutagenicity, antimutagenicity (Elgorashi *et al.*, 2003), Antibacterial, antihelmintic and cytotoxicity (McGaw *et al.*, 2007), antidiarrhoea (Galvez *et al.*, 1991), antibacterial (Eloff, 2001), anti-inflammation (Ojewole, 2010), antioxidant (Braca *et al.*, 2003), anti-diabetic (Ojewole, 2004)	Gallotannin, tannic, mallic, gallic and citric acid, triterpene, flavonoid, coumarins (Ojewole *et al.*, 2010)
Searsia gueinzii Sond (Syn *Rhus gueinii Sond*	Mushakaladza (V)	root	Gastrointestinal infections (Elgorashi *et al.*, 2003)	Mutagenicity , antimutagenicity (Elgorashi *et al.*, 2003)	-
Searsia incisa L.f.	uNongquthu	Root and bark decoction	Shock and diarrhoea (Dold and Cocks, 2001, McGaw *et al.*, 2008)		-
Searsia lancea L.f.	Mushakaladza (V)		Diarrhoea and gallsickness (Van der Merwe *et al.*, 2001; McGaw *et al.*, 2008)	Antibacterial, antihelminttic and cytotoxicity (McGaw *et al.*, 2007)	-
Searsia leptodictya Diels	Mushakaladza (V)	leaves	Browser, gall sickness in cattle, infectious disease, chest and abdominal pain (Sebothoma, 2010)	Antimicrobial (Sebothhoma, 2010)	(-)-leucofisetinidin, (-)-epicatechin and [4,8]-(+)-fisetinidol-(-)-epicatechin (Viviers *et al.*, 1983)
Searsia pendulina	-	Leaves	Stomach ailment, enema in children	-	-

Jacq.			(Coates-Palgrave, 2002)		
Searsia pentheri Zahlbr.	Muthasiri (V)	leaves	Epilepsy (Svenningsen *et al.*, 2006)	$GABA_A$/benzodiazepine receptor affinity (Svenningsen *et al.*, 2006)	Apeginin, agathisflavone (Svenningsen *et al.*, 2006)
Searsia rogersii Schonland	Muthasiri (V)	Bark	Pain, watery diarrhoea (Samie *et al.*, 2010)	Antifungal (Samie *et al.*, 2010), Antimycobacterium (Green *et al.*, 2010)	-
Annonaceae					
Annona senegalensis Pers.	Muembe (V)	Bark	Toothache, venereal, diarrhoea (Mabogo, 1990; More *et al.*, 2008)	Antidiarrhoeal (Suleiman *et al.*, 2008), antivenom (Adzu *et al.*, 2005), antimalaria (Okokon *et al.*, 2006)	**Annnosenegalina** (cytotoxic and antiparasitic**), Annonacin** (cytotoxic agent, insecticidal, mutagenic activity) immunosuppressant), **senegalene** (cytotoxic agent), **17, 19-kauranediol (*ent*-16β)-form. Dicarboxylic acid, 19-Methyl ester** (toxic to brime shrimp)
Uvaria chamae P. Beauv		Root	Catarrh, dysentery, fever, hematemesis, inflammation, jaundice, wounds, yellow fever (Reid et al., 2006)	Antimalaria (Okokon *et al.*, 2006); mutagenic and antimutagenic (Reid *et al.*, 2006)	-
Apiaceae					
Alepidia amatymbica Eckl. & Zeyh.	Iqwili, Ikhathazo (Z)	Root	Decoction for diarrhoea (Appidi *et al.*, 2008)	Antimicrobial, anti-inflammatory and genotoxicity (Mulaudzi *et al.*, 2009)	Rosmarinic acid, Dehydrokaurenoic aicd, Kaurenoic acid, kaurene lactone, acetoxy kaurene lactone (Holzapfel *et al.*, 1995)
Centella asiatica (L.) Urb.		Root	Chronic diarrhoea and dysentery; diaphoretic (van Wyk, 2008)	Modulator of nitric oxide production and TNF-α (Nhiem *et al.*, 2011), , lipid peroxidation (Kumar and Muller, 1999)	Asiaticoside G, asiaticoside, asiaticoside F, asiatic acid, quadranoside IV, 2a,3b,6b-trihydroxyolean-12-en-28-oic acid 28-O-[α-L-rhamnopyranosyl-(1→4)-β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl] ester, kaempferol, quercetin, astragalin, and isoquercetin (Nhiem *et al.*, 2011)
Centella glabrata L.		Root and stalk	Chronic diarrhoea and dysentery, diaphoretic (van Wyk, 2008b)	-	-
Foeniculum vulgare Mill.		Leaf	Flatulence, cough, diuretic, digestive problem, diarrhoea, stomach ache and cramps (Watt and Breeyer-Brandwijk, 1962; van Wyk, 1997)	Antimicrobial (Bacillus cereus, Clostridium botulinum, Salmonella enteritidis, Staphylococcus aureus, Yersinia enterocolitica) (Ceylan and Fung, 2004)	**Falcarindiol** (antifungal, antibiotic and analgesic, antinociceptive, DNA topoisomerase inhibitor, phytotoxic, allelochemical, antimutagenic and antiproliferative agents), 1-(4-hydroxyphenyl)-1,2-propanediol form, 4' methyl ether (phytotoxin, antiparasitic, nematocidal agent)
Apocynaceae					
Acokanthera oblongifolia (Hochst.) Codd	inHlungunyembe Intlungunyembe (X, Z)	leaves	Severe gastrointestinal irritation (Verschaeve and Van Staden, 2008), Decoction for stomach ache, diarrhoea (Bisi-Johnson *et al.*, 2010)	Genotoxicity (Elgorashi *et al.*, 2003); Epilepsy and convulsion (Risa *et al.*, 2004)	**Acolongifloroside K** and **H (**antineoplastic agent)
Catharanthus roseus (L.) G.Don	Imbali, Ikhwinini, Isishushlungu (Z)	Leaves, stem and	Diarrhoea (de Wet *et al.*, 2010)	Antimicrobial (van Vuuren and Naidoo, 2010)	**Serpentine** (antitumour activity);apparicine (cytotoxin, weak antbacterial, antiviral agent active against Polio virus,

		root			analeptic properties); **β-carboline** (induced mutagenicity, antiparasitic, antitrypanosomal agent); **Catharanthamine** (antitumour); **Trichosetin** (antibacteria); **16-Epi-2-isositsirikine** antineoplastic); **Leurosine** (antihyperglycaemic); **Lochnerinine** (antitumour); **Pericyclivine** (weak cytotoxic activity); **15',20'-anhydroviriblastine** (antineoplastic agent); **Vindoline** (antineoplastic); **Vindolinine** (antiglycaemic agent, antifungi); **Vingamine** (cytotoxic); **yohimbine** (selective α_2–adrenoceptor antagonist, antidepressant, antihypotensive, , antidiuretic activity, aphrodisiac, anxiogenic activity in rodent)
Sarcostemma viminale (L) R. Br subsp. *viminale*	Umbelebele, Ingotshwa	Stem	Infusion for diarrhoea (de Wet *et al.*, 2010), Increase livestock productivity (Kunene and Fossey, 2006)	Antibacterial, anti-inflammatory and mutagenic effects (Luseba *et al*, 2007)	-
Aquifoliaceae					
Ilex mitis (L.) Radlk.	Monamane (S), Mutanzwa-khamelo (V)	Root bark	Decoction for diarrhoea (Mathabe *et al.*, 2006)	Antimalaria and cytotoxicity (Rasoanaivo *et al.*, 2004)	-
Araliaceae					
Cussonia arborea Hochst ex A. Rich		Root, leaves	Decoction for diarrhoea (De Villiers *et al.*, 2010)	antimicrobial and antimalarial (De Villiers *et al.*, 2010)	Arboreaside A, Arboreaside B, Arboreaside C, Arboreaside D, Arboreaside E, ciwujianoside C3 and 23-hydroxyursolic acid 28-*O*-α-L-rhamnopyranosyl-(1→4)-*β*-D-glucopyranosyl-(1β6)-*β*-D-glucopyranosyl ester (Kougan *et al.*, 2009)
Asclepiadaceae					
Asclepias fruticosa L.	iGwada (X), Mutshulwa (V), Lebegana (S)	Root, leaves	Diarrhoea and stomach pain in children (Lewu and Afolayan, 2009)	Antimycobacterium (Green *et al.*, 2010), antifungal (Samie *et al.*, 2010), antimicrobial, anti-inflammatory, anticholinesterase and mutagenic activities (Ndhlala *et al.*, 2010)	-
Secamone filiformis (L.f) J. H. Ross	iMbijela	Stem	Diarrhoea in cattle (Dold and Cocks, 2001, McGaw *et al.*, 2008)	Antihelminthic, antibacterial and cytotoxicity (McGaw *et al.*, 2007)	-
Xysmalobium undulatum (L.) W.T. Aiton	Ishongwe (X, Z)	Roots	Diarrhoea , dysentery, stomach cramps, headache, oedema, dysmenorrhoea (Bisi-Johnson *et al.*, 2010)	Antibacterial (Rabe and Van Staden, 1997), PG inhibition (Jager *et al.*, 1996), Serotonin re-uptake modulatory activity (Nielsen *et al.*, 2004), antidepression (Pedersen *et al.*, 2008)	-

Asparagaceae					
Asparagus cooperi Bak.	Lefatshana (S)	Whole plant	Decoction for diarrhoea (Mathabe *et al.*, 2006)	Antibacterial (Mathabe *et al.*, 2006)	-
Asphodelaceae					
Aloe arborescens Miller	Inhlaba, Tshikhopha (V)	leaves	Diarrhoea and sore (Mlambo, 2008)	Anti-inflammatory (Lindsey *et al.*, 2002); immunomodulator, anti-inflammatory (Imanishi, 1993),	Aloctin A (Imanishi, 1993); aloenin, 2'-*O*-*p*-coumaroylaloesin, 2'-*O*-feruloylaloesin, isobarbaloin, and barbaloin (Beppu *et al.*, 2003)
Aloe candelabrum Berger	Ikhalana Inkalane (X) Uphondonde (Z)	leaves	Decoction for diarrhoea (Bisi-Johnson *et al.*, 2010)	-	-
Aloe greatheadii Schonl.	Sekgopha (S)	Leaves	Decoction for diarrhoea (Mathabe *et al.*, 2006)	antioxidant (Botes *et al.*, 2008), antiplasmodial and cytotoxicity (Van Dyk *et al*, 2009)	-
Aloe marlothii Berger	Bindamutsho, Tshikhopha (V)	Leaves	Gallsickness, parasites, diarrhoea, constipation, retain placenta, dystocia maggots (Van der Merwe *et al.*, 2001; McGaw *et al.*, 2008)	antimalaria (Pillay *et al.*, 2008), antibacterial, antihelminthic, anti-amoebic (McGaw *et al.*, 2000), antitick (Spicket *et al.*, 2007)	-
Bulbine abyssinica A. Rich	Utswelana Intelezi (X) Ibhucu (Z), Incelwane (X)	Leaves, tubers	Vomiting, diarrhoea, tuberculosis (Bisi-Johnson *et al.*, 2010)	Antileukemia, antiplasmodial, cytotoxicity (Bringmann *et al*, 2002)	Chrysophanol, aloe-emodin, knipholone, isoknipholone, Bulbine-knipholone (Bringmann *et al*, 2002)
Bulbine asphodeloides (L.) Willd		tuber and leaves	rashes, sores wounds, dysentery and diarrhoea (Iwalewa *et al.*, 2007)	-	-
Bulbine fructescen Wild	Intelezi (X)	leaf, root and rhizome	Diarrhoea, burns, rashes, blisters, insect bites, cracked lips and mouth ulcers (Coopoosamy, 2011)	Antibacterial (Coopoosamy, 2011), antiplasmodial (Mutanyatta *et al*, 2005)	knipholone, 4-O-demethylknipholone-4-β-D-glucopyranoside (Mutanyatta *et al*, 2005)
Bulbine latifolia (L.f) Roem et Schult	Irooiwater	Root	Decoction for diarrhoea (Appidi *et al.*, 2008)	Antibacterial (Coopoosamy, 2011)	**Knipholone** (antiplasmodial activity, cytotoxic agent)
Bulbine natalensis (Bak. Cf. roowortel	Ibhucu (Z)	leaves	Decoction for diarrhoea (Mathabe *et al.*, 2006); vomiting, diarrhoea, convulsion,venereal diseases, diabetes and rheumatism (Pujol, 1990)	Sexuality behaviour (Yakubu and Afolayan, 2008), Toxicity (Afolayan and Yakubu 2008)	-
Asteraceae					
Acanthospermum glabratum (DC) Wild	Inamathela	Whole plant	Diarrhoea (de Wet *et al.*, 2010)	-	-
Acanthospermum australe (Loefl.) O. Kuntze	Umgwaqeni (Z)	Whole plant	Diarrhoea (Mlambo, 2008)	Antiherpesvirus and antipoliovirus (Rocha Martin *et al.*, 2010)	Acanthoaustralide, quercitin and chrysosplenol (Rocha Martin *et al.*, 2010)

Artemisia absinthium L.		leaves	Diarrhoea (Van Wyk *et al.*, 2008)	Antimycobacterium (Gautam *et al.*, 2007)	-
Bidens bipinnata L.	Uvelemampo ndweni uvelegoli	leaves	Infusion for diarrhoea (Bisi-Johnson *et al.*, 2010), haemorrhage, reduce cancer, flu, cold, fever (Pooley, 1998)	Antidiarrhoea (Atta and Mouneir, 2005)	-
Bidens pilosa L.	iSanama, Mushidzhi (V)	Root or leaves, flowers	Stomach pain (Lewu and Afolayan, 2009); diarrhoea, inflammation, female infertility, excessive menstruation (Dold and Cocks, 2000)	Antidiarrhoeal (Yadav and Tangpu, 2009), amoebicidal (Moundipa *et al.*, 2005), immunomodulator (Chang *et al.*, 2007, Chiang *et al.*, 2007)	Centaurein, centaureidin, cytopiloyne (Chang *et al.*, 2007, Chiang *et al.*, 2007)
Brachylaena ilicifolia (Lam.) Phill. & Schweick	uMgqh	Leaves	Diarrhoea in lambs (Dold and Cocks, 2001; McGaw *et al.*, 2008)	-	-
Brachylaena transvaalensis E. Philips and Schweick	Iphahlalehlathi	Leaves and bark	Diarrhoea (de Wet *et al.*, 2010)	--	-
Callilepis laureola Hutch	Impila (Z)	Roots	Diarrhoea (Mlambo, 2008)	-	-
Chromolaena odorata L.	Usandanezwe (Z)	Leaves	Diarrhoea (Mlambo, 2008)	Anti-inflammatory, antipyretic antispasmodic (Taiwo *et al.*, 2000), antidiabetic (Wafo *et al.*,2011), antimicrobial and cytotoxicity (Vital and Rivera, 2009)	15-angeloyloxy-16,17-epoxy-19-kauronic acid, 16-kauren-19-oic acid, 6'-hydroxy-2',3',4,4'-tetramethoxychalcone, isosakuranetin, acacetin, and kaempferide (Wafo *et al.* 2011)
Conyza scabrida DC.		Herb	Cold, influenza, inflammation, diarrhoea, fever, diabetes, stomach affliction (Thring *et al.*, 2007)	Antimicrobial (Thring *et al.*, 2007)	-
Dicoma anomala Sond.	Umuna (Z), Inyongana (X)	Roots	Decoction for diarrhoea, stomach cramp and skin lesion (Shale *et al.*, 1999)	Antibacterial, antioxidant, fibroblast growth stimulant (Steenkamp *et al.*, 2004)	-
Dicoma capensis Less.		Herb	Bitter tonic and diuretic; kidney; bladder; back pain; nausea; influenza; colds; cancer; diarrhoea (van Wyk, 2008)	Cytotoxicity (Steenkamp and Gouws, 2006)	-
Helichrysum adenocarpum DC		Root decoction	Diarrhoea and vomiting in children (Lourens *et al.*, 2008)		-
Helichrysum calophyllum Klatt		Root	Hyperfunction of lower gastrointestinal tract (Lourens *et al.*, 2008)	-	-
Helichrysum		Root	Diarrhoea in children (Lourens *et al.*,	-	-

ecklonis Sond		decoction	2008)		
Helichrysum odoratissimum (L.)	Imphepho (Z)	Whole plant	Diarrhoea (Mlambo, 2008)	Antimicrobial (Puyvelde *et al.*, 1989)	3,5-dihydroxy-6,7,8-trimethoxyflavone and 3-0-methylquercetin, helichrysetin (Puyvelde *et al.*, 1989)
Pentzia incana (Thunb.) Kuntze			Diarrhoea (Van Wyk *et al.*, 2008)	-	-
Schkuhria pinnata (Lam.) Thell.	Unsakansaka (Z)	Aerial parts	Pneumonia, diarrhoea, eye infections, heartwater (Van der Merwe *et al.*, 2001; McGaw *et al.*, 2008)	Antibacterial , anti-inflammatory mutagenicity (Luseba *et al.*, 2007)	-
Senecio quinquelobus DC.	Usinini (Z)	Leaves	Diarrhoea (Mlambo, 2008)	-	-
Vernonia glaberrima Welw		Leaves	Decoction for diarrhoea (De Villiers *et al.*, 2010)	Antibacterial and antimalaria (De Villiers *et al.*, 2010)	-
Vernonia kotschyana Sch. Bip. ex Walp. (*Baccharoides adoensis* var. *kotschyana* (Sch. Bip. ex Walp.) M.A. Isawumi, G.El-Ghazaly & B. Nordenstam)	Inyathelo (Z)	leaves	Diarrhoea (Mlambo, 2008)	Immunomodualting activity (Nergard *et al.*, 2004); antibacterial activity (Deeni and Hussain, 1994)	pectic arabinogalactan (Nergard *et al.*, 2004)
Vernonia natalensis Sch. Bip. ex Walp.	Uhlambihloshane, Isibhaha	Leaves, stem	Decoction for stomach cramps, nervous spasms of the stomach (Fawole *et al.*, 2009b); (Hutching *et al.*, 1996)	Anti-inflammatory (Fawole *et al.*, 2009a), antimicrobial, mutagenicity (Fawole *et al.*, 2009b)	-
Vernonia oligocephala Sch. Bip	lihlunguhlungu	Roots	Infusion for diarrhoea (Amusan *et al.*, 2007)	-	-
Vernonia myriantha Hook. F (syn*Vernonia stipulacea* Klatt)	Mululudza (V)	Roots	Diarrhoea, fever, flu, contraceptive (Bessong *et al.*, 2005; Obi *et al.*, 2003)	-	-
Vernonia tigna Klatt syn *V. corymbosa*	Uhlunguhlungu (Z), Phathaphathane (V)	Leaves	Diarrhoea (Mlambo, 2008)	-	-
Balanitaceae					
Balanites maughamii Sprague		leaves	Diarrhoea in cattle (Luseba and Van der Merwe, 2006; McGaw *et al.*, 2008)	Antiplasmodial and cytotoxicity (Prozesky *et al.*, 2001)	-

Balanophoraceae					
Sarcophyte sanguine Sparrm		whole plant	Amenorrhoea, dysentery, diarrhoea and swellings growth (Iwalewa *et al.*, 2007)	Antibacterial and antifungal (Naidoo *et al.*, 1992)	Eriodictyol, naringenin, triandrin, n-pinitol (ID-4-O-methyl chiroinositol), trans-p-coumaraldehyde, Exocarpic acid (13E-octadecene-9,11-diynoic acid)
Bignoniaceae					
Markhamia sessilis Sprague		Leaves	Decoction for diarrhoea (De Villiers *et al.*, 2010)	Antiplasmodial and cytotoxicity (Mbatchi *et al.*, 2006), antimicrobial and antimalaria (De Villiers *et al.*, 2010)	-
Kigelia africana (Lam.) Benth.		Bark	Dysentery and stomach ailments (van Wyk, 2008b)	Antidiarrhoea (Akah, 1996), analgesic and anti-inflammatory (Owolabi and Omogbai, 2007), antifungal and antibacterial (Owolabi *et al.* 2007)	Verminoside and Verbascoside (Picerno *et al.*, 2005)
Tecomaria capensis Spach		Bark	fever, diarrhea and dysentery, pains, sleeplessness, stomach and chest pains (Iwalewa *et al.*, 2007)	Antimicrobial (Saini *et al.*, 2011)	-
Bombacaceae					
Adansonia digitata L.	Muvhuyu (V)	Leaves, bark, root fruit	Fever, diarrhoea, haemoptysis, hiccup remedy (van Wyk, 2008b)	Anti-inflammatory , antiviral (Selvarani and Hudson, 2009), antihyperglycermic and hypolipidermic (Bhargav *et al.*, 2009), Antimicrobial (Mulaudzi *et al.*, 2011)	Epicatchin, procyanidin B2, procyanidin B5 (Kinghorn *et al.*, 2011)
Bursareceae					
Commiphora harveyi (Engl.) Engl.		Leaves	Disinfectant for wound, anthelmintic and snakebite (Watt and Breyer-Brandwijk, 1962)	Antimicrobial (Suleiman *et al.*, 2010)	-
Capparaceae					
Capparis tomentosa	Umqoqolo (Z), Muoba-dali (V)	Root infusions and decoctions	Diarrhoea in cattle, stomach ailments in animals (Watt and Breyer-Brandwijk, 1962, Pujol, 1990, McGaw *et al.*, 2008)	Antimicrobial (Ramalivhana *et al.*, 2010), antifungal (Samie *et al.*, 2010)	**Stachydrine** L-form (Systolic depressant, rheumatism)
Caricaceae					
Carica papaya L.	Papawe (V)	Leaves, seed	Amoebic dysentery, fever, gastric problems, asthma, immune-stimulant (Green *et al.*, 2010; Aruoma *et al.*, 2006)	Antiamoebic (Tona *et al.*, 1998), anthelmintic (Kermanshai *et al.*, 2001)	**Alternariol Carpamine** (cardiotonic agent, CNS depressant), **Chymopapain**; **Glycerol triacetate** (antifunal and adjuvant); **Papain** ; **2,4'-Dihydroxy-3',5'-dimethoxyacetophenone** (antifungal), Benyl

					isothiocyanate (Kermanshai *et al.*, 2001)
Caryophyllaceae					
Krauseola mossambicina (Moss.) Pax & K. Hoffm.	Isihlaza, Isihlazi		Diarrhoea (de Wet *et al.*, 2010)	-	-
Celastraceae					
Elaeodendron transvaalense (Burtt Davy) R.H. Archer syn *Cassine transvaalensis*	Mulumanamana Mukuhazwhi, Umgugudo (Z)	Bark	Cough, piles, venereal diseases, diarrhoea, stomach ache, laxative (Samie *et al.*, 2010)	Antimicrobial (Tshikalange *et al.*, 2005), hypoglycaermic (Deutschlander *et al.*, 2009), Cytotoxicity (Tshikalange and Hussein, 2010)	lup-20(30)-ene-3,29-diol , lup-20(29)-ene-30-hydroxy-3-one-(2), ψ– taraxastanonol, β-sitosterol and 4' –O-methylepigallocatechin (Tshikalange and Hussein, 2010)
Gomphocarpus fruticosus Dryand.		Leaf infusion	Diarrhoea and stomach ache in children (Hutchings *et al.*, 1996; Fouche *et al.*, 2008)	-	**Gomphoside** (cardiotonic agent)
Gymnosporia senegalensis (Lam.) Loes	Ubuhlangwe		Diarrhoea (de Wet *et al.*, 2010)		-
Maytenus heterophylla Eckl. & Zeyh.) Robson	Isibhubu (Z), Tshiphandwa (V)	Bark and leaf infusions	Diarrhoea in stock animals (Watt and Breyer-Brandwijk, 1962; McGaw *et al.*, 2008)	Antimicrobial (Orabi *et al.*, 2001), anti-inflammatory and cytotoxicity (Da Silva *et al.*, 2010), anticytomegalovirus (Murayama *et al.*,2007)	1β-acetoxy-9α-benzoyloxy-2β,6α-dinicotinoyloxy-β-dihydroagarofuran, β-amyrin, maytenfolic acid, 3α-hydroxy-2-oxofriedelane-20α-carboxylic acid, lup-20(29)-ene-1β,3β-diol, (−)-4'-methyl*epi*gallocatechin, and (−)-*epi*catechin (Da Silva *et al.*, 2010) , pristimerin, lupeol and 2-acetylphenol-1-β-D-glucopyranosyl (1→6)-β-D-xylpyranoside (acetophenol glycoside) (Murayama *et al.*,2007)
Maytenus peduncularis (Sond.) Loes.	Mukwatule (V)	root	Backache, pain (Gonzalez *et al.*, 2000)	-	-
Maytenus procumbens (L.f.) Loes.	-	-	-	-	-
Maytenus senegalensis (Lam.) Exell	Tshiphandwa (V)		Root used for chest pain, rheumatism, snakebites, diarrhoea and fever. Leaves for eye infection (Matu and van Staden, 2003)	Antimicrobial and anti-inflammatory (Matu and van Staden, 2003); anti-inflammatory and cytotoxicity (Da Silva *et al.*, 2010)	**Wilforine** (insecticidal), β-amyrin, lupenone, maytenoic acid, β-sitosterol, pristimerin (Da Silva *et al.*, 2010)
Maytenus undata	Tshinembane (V)	Leaves		Antimicrobial, anti-inflammatory and	3-oxo-11α-methoxyolean-12-ene-30-oic acid, 3-oxo-11α-

(Thunb.) Blakelock				antioxidant (Muhammed *et al.*, 2000), antimalaria (Muthaura *et al.*, 2007)	hydroxyolean-12-ene-30-oic acid, 3-oxo-olean-9(11),12-diene-30-oic acid, 3,4-seco-olean-4(23),12-diene-3,29-dioic acid (20-epikoetjapic acid), 3,11-dioxoolean-12-ene-30-oic acid (3-oxo-18β-glycyrrhetinic acid), koetjapic acid, 12-oleanene artifact 3-oxo-11α-ethoxyolean-12-ene-30-oic acid (Muhammed *et al.*, 2000)
Chenopodiaceae					
Atriplex nummularia Lindl.		Laeves, flower	Diarrhoea (Van Wyk et al, 2008)	Antitumorigenic activity (Amara *et al.*, 2008)	-
Chenopodium ambrosioides L.	Unakani, Ikhambi	Aerial part	Diarrhoea (de Wet *et al.*, 2010)	Antisecretory against cholera toxin (Velazquez *et al.*, 2006), antiamoeba and antigardia (Calzada *et al.*, 2006)	Ascaridole, quercetin, kaempferol, isorhamnetin, ambroside, malic acid, succinic acid
Clusiaceae					
Garcinia livingstonei T. Anderson	Umphimbi, Muphiphi (V)	leaves	Diarrhoea (de Wet *et al.*, 2010)	Antibacterial (Kaikabo, 2008)	**Amentoflavone** (Bradykinin antagonist, anti-HIV activity, inhibitor of human cathepsin B, antiiflammatory properties), amentoflavone and 4"-methoxy amentoflavone (Kaikabo, 2008)
Combretaceae					
Combretum bracteosum (Hochst.) Brandis ex Engl.		leaves	-	anti-inflammatory, anthelmintic and antischistosomal (McGaw *et al.*, 2001)	-
Combretum imberbe Wawra	Mudzwiri (V)	Root	Decoction for diarrhoea (Mathabe *et al.*, 2006)	anti-inflammatory, anthelmintic and antischistosomal (McGaw *et al.*, 2001), antimicrobial (Angeh *et al.*, 2007)	1α, 23β-Dihydroxyl-12-Oleanen-29-oic acid-23β-O-α-4-acetylrhamnopyranoside; 1, 22-Dihydroxyl-12-Oleanen-30-oic acid; Ethyl cholesta-7, 22,25-trien-O-β-D-glucopyranoside (Angeh *et al.*, 2007), imberbic acid (Katerere *et al.*, 2003)
Combretum molle R. Br.ex G. Don	Mugwiti (V)	Roots	Abdominal pain, fever, snake bite, leprosy and convulsions (Bessong *et al.* ,2005; Mabogo, 1990)	anti-inflammatory, anthelmintic and antischistosomal (McGaw *et al.*, 2001)	Punicalgin, 4-*epi*-sericoside, sericoside (Asres *et al.*, 2001), β-D-glucopyranosyl 2α,3β,6β-trihydroxy-23-galloylolean-12-en-28-oate, combregenin, arjungenin, arjunglucoside I, combreglucoside (Ponou *et al.*, 2008), mollic acid glucoside (Oyewole, 2008)
Combretum padoides Engl. & Diels				anti-inflammatory, anthelmintic and antischistosomal (McGaw *et al.*, 2001); antifungal (Masoko *et al.*, 2007); Antibacterial (Angeh *et al.*, 2007)	1α,23β-dihydroxy-12-oleanen-29-oic-acid-23β-O-α-4-acetylrhamnopyranoside, 1,22-dihydroxy-12-oleanen-30-oic acid, 24-ethylcholesta-7,22,25-trien-O-β-D-glucopyranoside (Angeh *et al.*, 2007)
Combretum vendae A.E. van Wyk		Leaves	Leprosy, ophthalmic remedy, and blood purification (Watt and Breyer-Brandwijk,	Antimicrobial (Ahmed.*et al*, 2008; Suleiman *et al.*, 2010)	apigenin (Eloff *et al.*, 2008)

			1962)		
Combretum woodii Dummer				anti-inflammatory, anthelmintic and antischistosomal (McGaw *et al.*, 2001); antifungal (Masoko *et al.*, 2007)	Combretastatin B5 (Eloff *et al.*, 2005)
Combretum zeyheri Sond	Mufhatela-thundu, Mufhatela (V)	Root infusion	Bloody diarrhoea (Hutchings *et al.*, 1996; Fouche *et al.*, 2008)	Antibacterial (Breytenbach and Malan, 1989)	
Terminalia laxiflora Engl.		Leaves	Decoction for diarrhoea (De Villiers *et al.*, 2010)	Antifungal (Batawila *et al.*, 2005)	
Terminalia phanerophlebia Engl.		Root bark	Diarrhoea and colic (Iwalewa *et al.*, 2007)	Antimicrobial (Shai *et al.*, 2008a)	
Terminalia sericea Burch. ex DC.	Mususu (V), Ikonono	Leaves roots	Wound (Luseba and Van der Merwe, 2006); diarrhoea (Van der Merwe *et al.*, 2001; McGaw *et al.*, 2008)	Antimicrobial, antidiabetic, cytotoxicity (Moshi and Mbwambo, 2005), COX-1 and COX-2 assays (Eldeen *et al.*, 2006).	Anolignan B (Eldeen *et al.*, 2006), Termilignan B, Arjunic acid (Eldeen *et al.*, 2008), 3'5'-dihydroxy-4-(2-hydroxy-ethoxy) resveratrol-3-O-β-rutinoside, resveratrol-3-β-rutinoside glycoside, 3',4,5'-Trihydroxystilbene (resveratrol), arjungenin (Joseph *et al.*, 2007)
Convolvulaceae					
Ipomoea batatas (L.) Lam.	Sweet potato	Leaves	Decoction for diarrhoea (De Villiers *et al.*, 2010)		**4,5-Di-transcaffeoyldenoic acid** (antioxidant), **6-O-caffeoylsophorose** (α-glucosidase inhibitor, antioxidant); **3,5-Di-O-caffeoylquinic acid** (active against HIV-1 integrase, antiviral, antihepatotoxic activity); **Petrovin B** (antibacterial and antitumour)
Cornaceae					
Curtisia dentata (Burm.f.) C.A.Sm.= *C. faginea* Assegaai	Umlahleni (X, Z) Unsirayi (X), Umgxina	Bark, root	Diarrhoea, stomach ailments (Bisi-Johnson *et al.*, 2010)	Antimicrobial (Shai *et al* , 2008a, Shai *et al.*, 2009)	Lupeol, betulinic acid, ursolic acid, and 2α-hydroxyursolic acid (Shai *et al*, 2008b)
Crassulaceae					
Crassula ovata (Mill.) Druce	Karkay, karkey (K)	Fresh leave	Diarrhoea (van Wyk, 2008)	-	-
Crassula tetragona L.	Karkay, karkey (K)	Fresh leave	Diarrhoea (van Wyk, 2008)	-	-
Cucurbitaceae					
Cucumis hirsutus Sond.		Leaves, root	Decoction for abdominal pains, diarrhoea (Fawole *et al.*, 2009a); Hutching *et al.*, 1996)	-	-
Mormodica balsamina L.	Lubavhe (V)	Whole plant	Diabetes, childhood diarrhoea (Samie *et al.*, 2009)	Shigellocidal (Iwalokun *et al.* 2001); Cytotoxicity and antiamoebic (Samie *et al*, 2009)	Balsaminapentaol A, Balsaminol A, Balsaminol B, Cucurbalsaminol A, Cucurbalsaminol B, cucurbita-5,23(*E*)-diene-3*β*,7*β*,25-triol, karavilagenin E (Ramalhete

					et al.,(2009)
Ebenaceae					
Diospyros lycioides Desf.	Umbulwa (Z)	Bark, root	Decoction for bloody faeces and dysentery (Fawole *et al.*, 2009a); (Hutching *et al.*, 1996)	-	Hydroxyisodispyrin (cytotoxic agent)
Diospyros mespiliformis	Musuma	Bark and leaves	Dysentery, fever, ringworm, skin infection, wound healing (Samie *et al.*, 2010)		Diosquinone, plumbagin (Lajubutu *et al.*,1995)
Diospyros pallens (Thunb.) F. White		Root and stem	Stomach arch; diarrhoea (van Wyk, 2008)	-	-
Euclea crispa Thunb Gurke	Ungwali (Z),	leaves	Dysmenorrhoea (Steenkamp, 2003)		
Euclea natalensis A. DC	Mutangule-thavha (V), Umzimane (Z)	root	oral health care, for chest complains, bronchitis, pleurisy, chronic asthma, urinary tract infections, venearal diseases (Lall and Meyer, 2001) , Infertility and abortifacient (Arnold and Gulumian, 1984)	Antibacterial (Weigenand *et al.*, 2004), antimycobacterium (Lall and Meyer, 2001)	Octahydroeuclein, 20(29)-lupene-3β-isoferulate, shinanolone, lupeol, betulin (Weigenand *et al.*, 2004); diospyrin (Lall and Meyer, 2001)
Euphorbiaceae					
Antidesma venosum E. Mey. ex Tul.	Mupalakhwali (V)	Leaf	Decoction for abdominal cramps and dysentery (Fawole *et al.*, 2009a; Hutching *et al.*, 1996)	Antimicrobial (Fawole *et al.*, 2009b)	-
Bridelia micrantha (Hochst.) Baill	Munzere (V)	Bark, leaves, roots	Stomach ache, diarrhoea, abortifacient (Bessong *et al.*, 2005; Lin *et al.*, 2002), Gastro-intestinal ailments, painful joints, retained placenta, diabetes mellitus, syphilis prehepatic jaundice, tape worm abdominal pain, conjunctivitis, headache, scabies, bloody diarrhoea, dysentery, emetic, wound infection, coughs, threadworms, tonic for children, sore eyes, epigastric pain, relief of headache, purgative (Ngueyem *et al.*, 2009), diabetes mellitus (Abo et al., 2008)	Antidiarrhoea (Lin *et al.*, 2002), beta-lactamase inhibition (Gangoue-Pieboji *et al.*, 2007); antimalarial (Abo and Ashidi (1999). *n*-butanol fraction of methanolextract has IC50 of 7.3_g/ml against the RNA-dependent DNA polymerization (RDDP) function of HIV-1 RT (Bessong et al., 2006)	Taraxerol, gallic and ellagic acid, friedelin, delphinidin, methyl salicylate (Ngueyem *et al.*, 2009)
Bridelia mollis	Mukumbakumba	Leaves	Dysentery, burning and itching (Samie *et al.*, 2010)	Antifungal (Samie *et al.*, 2010)	
Euphorbia cooperi N. B. Br. Ex. Berger	Umhlonhlo (X)	Root bark	Diarrhoea, stomach disorder (Bisi-Johnson *et al.*, 2010)	-	-
Euphorbia hirta L.		Leaves	Decoction for diarrhoea (De Villiers *et al.*, 2010); dysentery, gonorrhoea, jaundice,	Antiamoebic , spasmolytic (Tona *et al.*, 2000), Antidiarrhoeal (Galvez *et al.*,	β-amyrin, 24-methylenecycloartenol, β-Sitosterol, Quercitrin (Galvez *et al.*, 1993). Quercitol, gallic acid,

			pimples, digestive problems and tumours, antibacterial, anti-inflammatory, antimalarial, galactogenic, antiasthmatic, antidiarrheal, anticancer, antioxidant, antiferlity, antiamoebic, and antifungal activities (Kumar *et al.*, 2010)	1993)	afzelin, quercitrin, myricitrin, rutin, gallic acid, quercitin, euphorbin-A and ephorbin-B, euphorbin-C, euphorbin-D, β-amyrin, 24-methylenecycloartenol, β-sitosterol, heptacosane, n-nonacosane, shikmic acid, tinyatoxin, choline, camphol, and quercitol (Kumar *et al.*, 2010)
Jatropha zeyheri Sond.	Xidomeja	Roots	General ailments, diarrhoea (Luseba and Van der Merwe, 2006; McGaw *et al.*, 2008)	Antimicrobial and Antifungal (Dekker *et al*, 1987)	Jaherin (Dekker *et al*, 1987)
Ricinus communis L.	Mupfure (T)	leaves	Wound and sores, asthma arthritis, flu, fever, tuberculosis, toothache, diarrhoea, antihelmentic (Bessong *et al.*, 2005; Grierson and Afolayan, 1999)	-	-
Spirostachys africana Sond	Morekhure (S)	Wood	Stomach ulcers, acute gastritis, headache, rashes, boil, emetic, purgative, diarrhoea, dysentery (Verschaeve and Van Staden, 2008)	Antibacterial and cytotoxicity (Mathabe *et al.*, 2008)	-
Fabaceae					
Acacia burkei Benth	umkhaya		Diarrhoea (de Wet *et al.*, 2010)	-	-
Acacia karoo Hayne	uMnga (X), Umunga (Z)	Bark, leaves	Diarrhoea, intestinal parasites in goats, sheep, poultry and pig (Dold and Cocks, 2001; McGaw *et al.*, 2008) fractures and diarrhoea (Van der Merwe *et al.*, 2001)	Anti-inflammatory (Adedapo *et al.*, 2008); Acute toxicity (Adedapo *et al*, 2008)	-
Acacia mearnsii De Wild Blackwood	Ublekwana (X) Udywabasi (X, Z) Indywabasi	Bark	Infusion for diarrhoea and dysentery (Bisi-Johnson *et al.*, 2010)	Protective against acrolein-induced oxidative damage (Huang *et al.*, 2010)	Robinetinidol-(4β→8)-epigallocatechin 3-O-gallate (Huang *et al.*, 2010)
Acacia robusta E. Meyer	Umngamanzi (Z)	leaves	Diarrhoea (Mlambo, 2008)	Antifungal (Hamza *et al.*, 2006)	
Acacia sieberiana DC.var *woodii* (Burtt Davy) Keay & Brenan	Musaunga, Muunga-luselo (V)	Bark	Enemas, antiseptic, fever, stomach ache, tapeworm, astringent, haemostatic, diarrhoea (Verschaeve and Van Staden, 2008)	Mutagenicity , antimutagenicity; antibacterial, antiinflammatory, anti-cholinesterase and mutagenic effects (Eldeen *et al.*, 2005)	-
Acacia tortilis (Forssk.) Hayne	Muunga-khanga, Muswu (V)		Diarrhoea (Van der Merwe *et al.*, 2001; McGaw *et al.*, 2008)		-
Bauhinia bowkeri Harv	uMdandlovu	Leaves, bark	Induce vomiting (Ndawonde *et al.*, 2007)	-	-
Bauhinia galpinii N. E. Br	Mutswiriri (V), Umhuwa (Z)	Bark, leaves	Diarrhoea, infertility (Samie *et al.*, 2010), infertility (Arnold and Gulumian, 1984),	Antimutagenic (Reid *et al.*, 2006); antioxidant and cytotoxicity of leaf	Quercetin-3-O-β-glactopyranoside, Myricetin-3-O-β-glactopyranoside, 2"-O-rhamnosylvitexin (Aderogba *et al.*,

			amenorrhoea (Van Wyk and Gericke, 2000)	extracts (Aderogba *et al.*, 2007); Anticampylobacterial, antiamoebic and cytotoxicity of root extract(Samie *et al.*,2009)	2007)
Bauhinia petersiana Bolle	Mushakule (V)	root	Cold (Coates-Palgrave, 2002); infertility and dysmenorrhoea (Van Wyk and Gericke, 2000)	-	-
Bauhinia variegata Linn		Leaves, bark	Diabetes, goiter, dysentery, diarrhoea (Parekh and Chanda, 2007)	Anti-inflammatory (Rao *et al.*, 2008); Immunomodulator (Ghaisas *et al.*, 2009)	kaempferol, ombuin, kaempferol 7,4'-dimethyl ether 3-O-β -D-glucopyranoside , kaempferol 3-O-β -D-glucopyranoside (4), isorhamnetin 3-O-β -D-glucopyranoside, hesperidin, 3β-trans-(3,4-dihydroxycinnamoyloxy)olean-12-en-28-oic acid (Rao *et al.*, 2008)
Dichrostachys cinerea (L.) Wight and Am.	Murenzhe (V)	Bark	Diarrhoea and steaming to get ride of acne (Mlambo, 2008)	spasmodic in guinea-pig isolated trachea (Aworet-Samseny *et al.*, 2011)	dichrostachines A-R (Long *et al.*, 2009)
Elephantorrhiza burkei Benth.	Umdabu (Z), Tshisese-thavha, Tshisesevhufa (V)	root	abdominal pains, diarrhoea, coughs, bacterial infections (Iwalewa *et al.*, 2007)	Antimicrobial (Mathabe *et al.*, 2006)	Triterpenoids, α-amyrim, β-sitosterol, alkaloids and saponin
Elephantorrhiza evoluta (Burch.) Skeels	iNtololwane (X, Z)	Roots, aerial part and bulb	Diarrhoea and dysentery in cattle, horse and humans (Watt and Breyer-Brandwijk, 1962; McGaw *et al.*, 2008)	-	-
Elephantorrhiza elephantina (Burch.) Skeels	Leshitsana	Stem rhizome	Decoction for diarrhoea (Mathabe *et al.*, 2006)	Antimicrobial (Mathabe *et al.*, 2006), antiparasitic (Naidoo *et al.*, 2006), antibabesia (Naidoo *et al.*, 2005	
Eriosema psoraleoides (Lam.) G. Don		Leaves	Decoction for diarrhoea (De Villiers *et al.*, 2010)	Antimicrobial (Khan *et al.*, 2000)	
Erythrina latissima E. Mey	Muvhale (V)		Sores (Coates-Palgrave, 2002)		erysotrine, erysodine, syringaresinol, vanillic acid, (+)-10,11-dioxoerysotrine, 2-(5'-hydroxy-3'-methoxy phenyl)-6-hydroxy-5-methoxybenzofuran, 7,3'-dihydroxy-4'-methoxy-5'-(γ,γ-dimethylallyl)isoflavone (erylatissin A) (Wanjala *et al.*, 2002), 7,3'-dihydroxy-6",6"-dimethyl-4",5"-dehydropyrano [2",3": 4',5']isoflavone (erylatissin B), (−)-7,3'-dihydroxy-4'-methoxy-5'-(γ,γ dimethylallyl)flavanone (erylatissin C) (Chacha *et al.*, 2004)
Indigofera daleoides		Whole plant	Decoction for diarrhoea (Mathabe *et al.*,	Antimicrobial (Mathabe *et al.*, 2006)	(6,2-O-[3-nitropropanoyl-β-D-glucopyranose]), (6,3',4'-

Benth. ex Harv & Sond			2006)		trihydroxyflavan 5'-O-glucopyranoside) (Mathabe *et al.*, 2009)
Indigofera jucunda Schrire syn *Indigofera cylindrical sensu* E. Mey		Root	Intestinal worm (Coates-Palgrave, 2002)	-	-
Indigofera sessilifolia DC.	iKhubalo	Roots	Diarrhoea in calves (Dold and Cocks, 2001; McGaw *et al.*, 2008)	-	-
Mucuna coriacea Baker	Vhaulada	Roots	Fever, diarrhoea (Bessong *et al.*, 2005)	Antimicorbial (Samie *et al.*, 2009)	N.A
Peltophorum africanum Sond.	Musese (T)	Bark , root bark	Tonic, diarrhoea (Van der Merwe *et al.*, 2001; McGaw *et al.*, 2008)	Anti parasitic (Bizimenyera *et al.*, 2006), anti HIV (Bessong *et al.*, 2005)	Catechin, gallotannin, bergenin (Bessong *et al.*, 2005)
Rhynchosia adenoids E. & Z.		Roots	Decoction for rheumatic pains, menstrual pains and dysentery (Shale *et al.*, 1999)	Cyclooxygenase inhibitory (Jager and Van Staden, 2005)	
Senna italic Mill.	Ximbangam bangana	Bark, roots	Diarrhoea and gallsickness diarrhoea, (Luseba and Van der Merwe, 2006; McGaw *et al.*, 2008)	-	--
Senna occidentalis (L) Link	Ikhoshokhosho	Leaves, root	Diarrhoea (de Wet *et al.*, 2010)		
Schotia brachypetala Sond.	Mulubi (V)	Bark	Diarrhoea (Mathabe *et al.*, 2006), root for dysentery and diarrhoea (Hutching *et al.*, 1996)	Antibacterial (McGaw *etal.*, 2002)	Linolenic acid and methyl-5,11,14,17-eicosatetraenoate (McGaw *etal.*, 2002)
Schotia latifolia Jacq.	Umgxam	bark	Decoction for diarrhoea (Appidi *et al.*, 2008)	Antibacterial (Masika *et al.*, 2004)	Epicathechin and catechin (Masika *et al.*, 2004)
Zornia milneana	Lukandulula (V)	Whole plant	Dysentery and diarrhoea (Samie et al., 2005)	Anticampylobacterial and antiamoebic (Samie *et al.*,2009)	-
Flacourtiaceae					
Oncoba spinosa Lam		root	Dysentery bladder problem (Verschaeve and Van Staden, 2008)	Mutagenicity , antimutagenicity, Epilepsy and convulsion (Risa *et al.*, 2004)	-
Gentianaceae					
Chironia baccifera L.			treat acne, sores and diarrhoea (Watt and Breyer-Brandwijk, 1962; van Wyk *et al.*, 1997)	Antibacterial (Thring *et al.*, 2007)	-
Geraniaceae					
Geranium incanum Burm. f.	Isikhwali (Z)	leaves	Diarrhoea (Amabeoku, 2009; Van Wyk *et al.* 1997)	Antidiarrhoearic (Amabeoku, 2009); antimicrobial and cytotoxicity (Babajide	-

				et al., 2010)	
Monsonia emarginata (L.f.) L'Hèr.		Herb and root	Diarrhoea, dysentery, cold and inflammation (van Wyk, 2008)	-	-
Monsonia burkeana Planch. Ex Harv.		Herb and root	Diarrhoea, dysentery, cold and inflammation (van Wyk, 2008)	Antioxidant (Mamphiswana *et al.*, 2010)	-
Pelargonium antidysentericum (Eckl.& Zeyh.) Kostel		Tubers	Used as astringent, diarrhoea, dysenteric fever (Brendler and van Wyk, 2008)	-	-
Pelargonium luridum (Andr.) Sweet	Umsongelo (X) ishwaqa	Leaf, root	Diarrhoea, dysentery, fever and colic (Brendler and van Wyk, 2008)	-	-
Pelargonium reniforme Curtis	iNtololwana, uVendle	Tuberous root	Diarrhoea and dysentery (van Wyk, 2008)	Antibacterial, antifungal and antioxidant (Adewusi and Afolayan, 2009a), Acute toxicity (Adewusi and Afolayan, 2009b)	scopoletin, umckalin, 5,6,7-trimethoxycoumarin, 6,8-dihydroxy-5,7-dimethoxycoumarin, (+)-catechin, gallic acid (Kayser and Kolodziej, 1997)
Pelargonium sidoides DC.	Umsongelo (X)		Tuberculosis, diarrhoea (Brendler and van Wyk, 2008)	Immunomodulatory (Kayser *et al.*, 2001), antibacterial, antifunfal and antitubercular (Mativandlela *et al.*, 2006)	scopoletin, umckalin, 5,6,7-trimethoxycoumarin, 6,8-dihydroxy-5,7-dimethoxycoumarin, (+)-catechin, gallic acid (Kayser and Kolodziej, 1997)
Pelargonium triste (L.) L'Hèr.		Tuberous root	Diarrhoea and dysentery (van Wyk, 2008)	-	-
Hyacinthaceae					
Eucomis autumnalis (Mill.) Chitt.	Ubuhlungu becanti Isithithibala (X) Umathunga (Z)	Bulb	Stomach ache, diarrhoea, back pain, healing of fractures (Bisi-Johnson *et al.*, 2010)	Anti-inflammatory (Zschocke et al., 2000)	-
E. regia (L.) L'Herit			Veneral diseases, lumbago, diarrhoea, respiratory conditions especially coughs, biliousness and to prevent premature childbirth (Watt and Breyer-Brandwijk, 1962)	COX-1 assay (Taylor and van Staden, 2001)	-
Ledebouria revoluta (L.f.) Jessop	iKreketsana (X)	Bulb	Bulb infusion for diarrhoea in goat, leaf decoction for gallsickness (Dold and Cocks, 2001; McGaw *et al.*, 2008)		(3R)-5,7-dihydroxy-3-(4'-methoxybenzyl)-4-chromanone, (3R)-5,7-dihydroxy-3-(4'-hydroxybenzyl)-4-chromanone, 3R)-5-hydroxy-7,8-dimethoxy-3-(4'-hydroxybenzyl)-4-chromanone, (3R)-5,7-dihydroxy-8-methoxy-3-(4'-hydroxybenzyl)-4-chromanone (Moodley *et al.*, 2006)

Schizocarphus rigidofolius	Ingcino (S)	leaves	Infusion for diarrhoea (Amusan *et al.*, 2007)	-	-
Scilla nervosa (Burch.) Jessop	Umagaqana, magaqana (X) Imbizankulu ingema (Z)	Root, bulb	All purpose herb. Diarrhoea, tuberculosis (Bisi-Johnson *et al.*, 2010)	-	-
Hydnoraceae					
Hydnora africana	Ubuklunga (X) Umavumbuka (Z) Umafumbuka (X)	Fruits, tuber leaves	Diarrhoea, dysentery (Bisi-Johnson *et al.*, 2010)	-	-
Hypoxidaceae					
Hypoxis latifolia Hook.	Inongwe Ilabateka (X)	Tuber	Decoction for diarrhoea (Bisi-Johnson *et al.*, 2011)	Antibacterial, antifungal (Buwa and Van Staden, 2006)	
Hypoxis hemerocallidea Fisch. & C. A. Mey	Inongwe Ilabateka (X)	Tuber	Decoction for diarrhoea (Ojewole *et al*, 2009)	Antinociceptive, anti-inflammatory and antidiabetic (Ojewole, 2006), Antidiarrhoeal, acute toxicity test (Ojewole *et al*, 2009)	
Iridaceae					
Crocosmia paniculata (Klatt.) Goldbl.	Undwendweni (Z)	corms	Diarrhoea in bovine (Watt and Breyer-Brandwijk, 1962; McGaw *et al.*, 2008)	-	-
Gladiolus dalenii van Geel		corm	Dysentery, diarrhoea, stomach cramps (Fawole *et al.*, 2009a; Hutching *et al.*, 1996)	Anti-inflammatory (Fawole *et al.*, 2009a), amoebicidal (Moundipa *et al.*, 2005), Antimicrobial and mutagenicity (Fawole *et al.*, 2009b)	-
Gladiolus sericeo-villosus Hook. F	Umnunge (X), Umlunge (Z)	Corm	Decoction fro dysentery, cold, tuberculosis diarrhoea (Bisi-Johnson *et al.*, 2010)	-	-
Watsonia densiflora Bak.		Corm	Diarrhoea in calves (Watt and Breyer-Brandwijk, 1962; McGaw *et al.*, 2008)	Antibacterial, antifungal, acetylcholinestarase inhition, mutagenicity, COX 1and 2 (Ndhala *et al.*, 2010)	
Watsonia tabularis Bak		corm	Diarrhoea in human and calves (Fawole *et al.*, 2009a; Hutching *et al.*, 1996)	Antimicrobial and mutagenicity (Fawole *et al.*, 2009b)	
Lamiaceae					
Ballota africana (L.) Berth.		herb	Stomach disorders, colds, liver complains (Thring and Weitz, 2006)		
Cissus quandrangularis	Isinwasi (Z), Nyangala (T)	Root, stem	Burns, wounds, gastrointestinal complaints, backache, body- and febrile pain, malaria (Lin *et al.*, 1999; Hutchings et al., 1996)	Antibacterial, anti-inflammatory and mutagenicity (Luseba, *et al.*, 2007)	

(Linn)					
Leonotis leonurus (L.) R.Br	Imunyamunya (Z)	leaves and stem bark	feverish headache, dysentery, coughs and colds, and haemorrhoids (Iwalewa *et al.*, 2007)	Anticonvulsant (Bienvenu *et al.*, 2002), antinociceptive, anti-inflammatory and hypoglycaemic activities (Ojewole, 2005)	1,2,3-trihydroxy-3,7,11,15-tetramethylhexadecan-1-yl-palmitate, succinic acid, uracil, luteolin 7-O-glucoside, acteoside, geniposidic acid (Agnihotri *et al.*, 2009)
Leucas capensis (Benth.) Engl.	uPhiphiyo	leaves	Decoction with *Aloe forex* and *Brachylaena ilicifolia* for diarrhoea in lambs (Dold and Cocks, 2001)	-	-
Mentha longifera (L.) L.		Leaf	Anti-diarrhoea (Naseri *et al.*, 2008)	Spasmolyte (Naseri *et al.*, 2008)	-
Rotheca myricoides (Hochst.) Steane & Mabb.		Root bark	Fever and diarrhoea in cattle (Verschaeve and Van Staden, 2008)	Mutagenicity and antimutagenicity (Verschaeve and Van Staden, 2008)	-
Salvia africana-caerulea L.		Leaf	Coughs, colds, women ailments; diarrhoea (van Wyk, 2008)	Antimicrobial, antioxidant, anti-inflammatory, antiplasmodial, cytotoxicity and antituberculosis (Kamatou *et al.*, 2006)	-
Salvia repens Burch. Ex. Benth		Roots, leaves, whole plant	Sores on the body, stomach problems, diarrhoea (Kamatou *et al.*, 2008)	Antimicrobial, antioxidant, anti-inflammatory, antiplasmodial, cytotoxicity and antituberculosis (Kamatou *et al.*, 2006)	-
Tetradenia riparia (Hochst.) Codd	Iboza (Z)	leaves	Cough, sore throats, malaria, dengue, dropsy, fever, diarrhoea, haemoptysis, boils, mumps, induce drowsiness (Verschaeve and Van Staden, 2008)	Mutagenicity , antimutagenicity (Verschaeve and Van Staden, 2008); Antibacterial, antifungal, acetylcholinestarase inhition, mutagenicity, COX 1and 2 (Ndhala *et al.*, 2010)	-
Teucrium riparium Hochst	umnunu	Root	Infusion for diarrhoea (Amusan *et al.*, 2007)	-	-
Lauraceae					
Ocotea bullata (Burch.) Baill.		Bark	Headache, infantile diarrhoea, stomach problems, emetic for emotional and nervous disorder (Verschaeve and Van Staden, 2008)	Mutagenicity , antimutagenicity (Verschaeve and Van Staden, 2008), anti-inflammatory (Zschocke *et al.*, 2000)	Ocobullenone, iso-ocobullenone, sibyllenone (Zschocke *et al.*, 2000)
Loganiaceae					
Strychnos henningsii Gilg.	uMnonono, Umqalothi (Z)	Bark infusion	Heartwater and diarrhoea in cattle (Dold and Cocks, 2001; McGaw *et al.*, 2008)	-	-
Loganiaceae					

Sida alba Forrsk		Leaves	Diarrhoea and dysentery (Samie et al., 2005)	Antibacterial (Samie et al., 2005)	-
Malva parviflora L.	Ujongilanga	leaves	Decoction for diarrhoea (Appidi *et al.*, 2008)	Antibacterial and anti-inflammatory (Shale *et al.*, 2005)	-
Melastomataceae					
Dissotis princeps (Kunth) Triana		Leaves	Infusion for diarrhoea and dysentery (Fawole *et al.*, 2009a; Hutching *et al.*, 1996)	Anti-inflammatory (Fawole *et al.*, 2009a), Antimicrobial and mutagenicity (Fawole *et al.*, 2009b)	-
Meliaceae					
Ekebergia capensis Sparrm		Root, bark	Stomach and intestinal complaints, dysentery, heart burn, purgative, kidney problem, indigestion (Verschaeve and Van Staden, 2008)	Mutagenicity , antimutagenicity (Verschaeve and Van Staden, 2008), antiplasmodial (Murata *et al.*, 2008)	Ekersenin, 4,6-dimethoxy-5-methylcoumarin, oleanolic acid, 3-epioleanolic acid, oleanoic acid (15), 3,11-dioxoolean-12-en-28-oic acid, melliferone, 3-oxo-11,13(18)-oleandien-28-oic acid, ekeberin A, (Z)-volkendousin, ekeberin B, 7-deacetoxy-7-oxogedunin, 7-acetylneotrichilenone, proceranolide, mexicanolide, swietenolide, methylangolensate, ekeberins C1, C2, and C3, 2,3,22,23-tetrahydroxy-2,6,10,15,19,23-hexamethyl-6,10,14,18-tetracosatetraene (3R,22R), 2-hydroxymethyl-2,3,22,23-tetrahydroxy-2,6,10,15,19,23-hexamethyl-6,10,14,18-tetracosatetraene (2R,3R,22R), ekeberins D1, D2, D3, D4, and D5 (Murata *et al.*, 2008)
Melia azedarach L.	Umsilinga (Z)	Leaves	Diarrhoea (de Wet *et al.*, 2010)		
Trichilia dregeana Sond.	Umkhuhlu (Z)	Leaves		antibacterial, antiinflammatory, anti-cholinesterase and mutagenic effects (Eldeen *et al.*, 2005)	cycloart-23-ene-3,25-diol (Eldeen *et al.*, 2007)
Trichilia emetica Vahl.	Umkhuhlu (Z)	Leaves	Diarrhoea (de Wet *et al.*, 2010)	Antimicrobial, antioxidant, anti-inflammatory, antimalarial, cytotoxicity (Komane et al., 2011)	sendanin, trichilinin, trichilin A, trichilin B, trichilin C, trichilin D, trichilin E, dregeana, nymania 1, rohituka, rohituka, rohituka, Trichilia substance Tr-A, Trichilia substance Tr-B, Trichilia substance Tr-C and seco-A-protolimonoid (Komane et al., 2011)
Menispermaceae					
Albertisia delagoensis (N.E. Br.) Forman	Umgandanganda ,ungandingandi	Root	Diarrhoea , dysentery, cough, colic, bloody stool (De Wet and van Wyk, 2008)	Antiplasmodial and cytotoxicity (De wet *et al.*, 2007)	
Antizoma angustifolia (Burch.) Miers ex Harv		Root	Diarrhoea , dysentery, cough, colic, bloody stool (De Wet and van Wyk, 2008)	-	-
Cissampelos	Umbombo (Z)	Root,	Purgative, tincture for dysentery (van Wyk,	-	-

capensis (L.f.) Diels		rhizome	2008)		
Cissamperos hirta Klotzch	Umanyokane, khalimelo (Z)		Diarrhoea (de Wet *et al.*, 2010)	-	-
Cissampelos mucronata A. Rich.		Root	Diarrhoea (Giess and Snyman, 1986; Von Koenen, 2001)	Anti-ulcer (Akah and Nwafor, 1999), sedative (Akah *et al.*, 2002), Antiplasmodial (Tshinbagu *et al.*, 2003)	Bisbenzylisoquinone alkaloid (Tshinbagu *et al.*, 2003)
Cissampelos torulosa E.Mey	Lukandulula (V)	Leaves	Diarrhoea and dysentery, sore throat (Mabogo, 1990; Samie et al., 2005)	Antiamoebic (Samie *et al.*, 2009), antibacterial (Samie *et al.*, 2005)	-
Moraceae					
Ficus capensis Thunb.	Infusion	Fruit	Diarrhoea (Pallant and Steenkamp, 2008)	intestinal motility modulation (Ayinde and Owolabi, 2009)	-
Ficus craterostoma Mildbr. & Burret	Muumo (V), inTendekwane, umThombe(X)		Stomach-ache (Bhats and Jacobs , 1995)	-	-
Ficus glumosa Delile		Bark	Decoction for diarrhoea (Venter and Venter , 2002)	-	-
Ficus sur Forssk	Umkhiwane (Z)	Leaves	Diarrhoea (Mlambo, 2008)	Spasmolytic and gastrointestinal protection (Kunle *et al.*, 1999)	-
Myrtaceae					
Psidium guajava L.	Ugwava (X, Z)	Leaves	Infusion for bloody diarrhoea (Bisi-Johnson *et al.*, 2010)	Antidiarrhoeal Tona et al., 1999; Lutterodt, 1992); antispasmolytic (Conde et al., 2003), antirotavirus (Goncalves et al., 2005), antimicrobial intestinal adhesion (Coutino et al., 2001)	Limonene, copaene, Asiatic acid, β-carotene, morin-3-O-α-L-arabinopyranoside, avicularin, gauijaverin, quercitin ellargic acid (Gutierrez *et al.*, 2008)
Syzygium cordatum Hochst. Ex. C. Krauss	Umdoni, Mutu (V)	Leaves, bark	Respiratory disorders, tuberculosis, stomach complaints, emetics, diarrhoea, cold, fever (Verschaeve and Van Staden, 2008)	Mutagenicity , antimutagenicity (Verschaeve and Van Staden, 2008); antimycobacterium (Mativandlela *et al.*, 2008); Antiescherichia (Sibandze *et al.*, 2010)	-
Syzygium paniculatum Gaertner	-	-	-	--	-
Olacaceae					
Ximenia caffra Sond	Mutswili (V)	leaves	Diarrhoea and dysentery (Green *et al.*, 2010; Fabry *et al.*, 1996)	Antigardial (John *et al.*, 1995), Antiamoebic (Samie *et al.*, 2009), antifungal (Samie *et al.*, 2010)	

Punica granatum L.	iRharnathi (X)	Fruit rind, roots	Diarrhoea and dysentery (van Wyk, 2008; Dold and Cocks, 2000)	Antidiarrhoeal (Pillai, 1992; Qnais *et al.*, 2007), Anti-inflammatory (Lansky and Newman, 2007)	Ellagitannins, anthocyanins, flavone glucosides, flavones, flavonol, flavonol glucosides, hydroxycinnamic acid, hydroxybenzoic acid, flavan-3-ols, alkaloids, sterol, triterpenoids (Lansky and Newman, 2007)
Oleaceae					
Olea europaea L. Subsp africana (Mill.) P.S.Green	Uzintlwa (X), uMnquma (X)		Anti-hypertensive, diuretic, tonic, diarrhoea, sore throat (Amabeoku and Bamuamba, 2010)	Antidiarrhoeal (Amabeoku and Bamuamba, 2010)	-
Orchidaceae					
Polystachya ottoniana Rchb.f.			Diarrhoea (Chinsamy *et al.*, 2010)	-	-
Pedaliaceae					
Ceratotheca triloba (Bernh.) Hook	Udonqabathwa (Z)	Leaf	Infusion for diarrhoea and gastrointestinal cramps (Watt and Breyer-Brandwijk, 1962; Roberts, 1990)	5-lipooxygenase inhibitory and antioxidant (Akula and Odhav, 2008), α-amylase inhibitory (Odhav *et al.*, 2010).	-
Plantaginaceae					
Plantago major L.		Seed, root	Diarrhoea (van Wyk, 2008)	Antidiarrhoeal (Atta and Mouneir, 2005), Hepatoprotective and anti-inflammatory (Turel *et al.*, 2007), wound healing activity, anti-inflammatory, analgesic, antioxidant, weak antibiotic, immuno modulating and antiulcerogenic activity (Samuelsen, 2000)	Indicain, plantagonin, baicalein, hispidulin, plantaginin, aucubin, fumaric acid, syringic acid, vanillic acid, *p*-hydroxy benzoic acid, ferulic acid, *p*-coumaric acid, gentisic acid, salicylic acid, benzoic acid, cinnamic acid oleanolic acid, ursolic acid, 18β-glycyrrhetinic acid and sitosterol (Samuelsen, 2000)
Plantago lanceolata L.		Seed	Diarrhoea (van Wyk, 2008)	-	-
Plumbaginaceae					
Plumbago auriculata	uTshintshini	Roots	Diarrhoea in cow (Dold and Cocks, 2001; McGaw *et al.*, 2008)	Antibacterial and anti cancer (Bisi-Johnson *et al.*, 2011)	-
Polygonaceae					
Rumex lanceolatus Thunb	Idololenkonyane (Z), Idolonyana (X)	Roots	Infantile diarrhoea, tapeworm, wound and sores (Dold and Cocks, 2000)	-	-
Rumex obtusifolius	Idololenkonyane (X, Z)	leaves	Diarrhoea (Bisi-Johnson *et al.*, 2010)	-	-
Portulaceae					
Portulacaria afra Jacq.	Umndibili (Z)	Leaves	Diarrhoea (Mlambo, 20080	-	-

Proteaceae					
Protea caffra Meisn.	Tshidzungu (V)	Root bark decoction	Calves with bloody diarrhoea (Hutching *et al.*, 1996)	-	-
Protea nitida Mill.		Bark	Astringent for diarrhoea (van Wyk, 2008)	-	-
Protea simplex		Root, bark	Decoction and infusion for diarrhoea, dysentery, stomach pain in human (Fawole *et al.*, 2009a; Hutching *et al.*, 1996)	Anti-inflammatory (Fawole *et al.*, 2009a), Antimicrobial and mutagenicity (Fawole *et al.*, 2009b)	-
Protea welwitschii Engl.			Dysentery, diarrhoea in calves and humans (Watt and Breyer-Brandwijk, 1962; McGaw *et al.*, 2008)	-	-
Punicaceae					
Punica granatum L.	Mokgranata	Root	Decoction for diarrhoea (Mathabe *et al.*, 2006)	-	-
Rhamnaceae					
Ziziphus mucronata Willd.	Mukhalu, Mutshetshete (V)	Leaves, bark, roots	Boils, sores, grandular swelling, diarrhoea, dysentery, cough (Verschaeve and Van Staden, 2008; Green *et al.*, 2010)	Anti-inflammatory (Fawole *et al.*, 2009a), Antimicrobial and mutagenicity (Fawole *et al.*, 2009b)	-
Ziziphus zeyheriana Sond.		Root-stock	Diarrhoea, internal parasites, general ailments (Van der Merwe *et al.*, 2001; McGaw *et al.*, 2008)	-	-
Rosaceae					
Prunus africana (Hook.f) Kalkman Red Stinkwood	Umkhakhazi (X), Umkakase (X)	Root	Diarrhoea, abdominal ailments (Bisi-Johnson *et al.*, 2010)		
Prunus persica (L.) Batsch.	Ipesika	Leaf decoctions	Diarrhoea in lamb and kid goats (Dold and Cocks, 2001; McGaw *et al.*, 2008)		
Rubiaceae					
Breonadia salicina (Vahl) Hepper & J. R. I. Wood		Bark decoctions	Diarrhoea, bloody stool, colic (Neuwinger, 1996; Venter and Venter, 2002)	Antiescherichial (Sibandze *et al.*, 2010)	
Nauclea latifolia Smith		Root	Dysentry, dyspepsia, fever, gastritis, gonorrhea, malaria,leprosy, measles, piles, toothache (Reid *et al.*, 2006)	Antiamoebic (Tona *et al.*, 1998; Moundipa *et al.*, 2005); antidiarrhoeal (Owolabi *et al.*, 2010)	
Pentanisia prunelloides (Klotzsch exEckl. & Zeyh) Walp	Icishamlilo, Icimamilo (X, Z)	Root, leaves, bulb	Vomiting, diarrhoea in children((Bisi-Johnson *et al.*, 2010)	Antibacterial (Yff *et al.*, 2002)	Palmitic acid (Yff *et al.*, 2002)

Psychotria capensis (Eckl.) Vatke	Ishithitibala (Z), UmGono-gono (X)	Fruits	Diarrhoea and vomiting (Bisi-Johnson *et al.*, 2010)	-	-
Rubia petiolaris DC.		Root	Diarrhoea and dysentery (van Wyk, 2008)	-	-
Rubus pinnatus Willd.	iQunube	Roots	Diarrhoea, haemorrhoids, epilepsy (van Wyk, 2008)	-	-
Rubus rigidus		Root	Diarrhoea and dysentery, toothache, coughs and colds (Iwalewa *et al.*, 2007).	-	-
Vangueria infausta Burch. subsp. infausta	Umviyo		Diarrhoea (de Wet *et al.*, 2010)	Antibacterial and antifungal (de Boer *et al.*, 2005)	-
Rutaceae					
Agathosma betulina (Bergius) Pillans			Antispasmodic, antipyretic, cough, Kidney and urinary tract infection, cholera and stomach ailment (Molla and Viljoen, 2008)	Antidiarrhoea and antibacterial (Lis-Balchin *et al.*, 2001); anti-inflammatory and antioxidant (Steenkamp *et al.*, 2006)	-
Agathosma crenulata (L.) Pillans			Antispasmodic, antipyretic, cough, Kidney and urinary tract infection, cholera and stomach ailment (Molla and Viljoen, 2008)	Antidiarrhoea and antibacterial (Lis-Balchin *et al.*, 2001)	-
Rutaceae					
Clausena anisata (Willd.) Hook.F. Ex Benth.		Bark infusion	Dysentery in cattle (Hutching *et al.*, 1996)	-	-
Ruta graveolens L.	iVendrit (X)	Leaves	Fever, convulsion, epilepsy, diarrhoea, cardiac asthma, jaundice (Dold and Cocks, 2000)	-	-
Sapindaceae					
Hippobromus pauciflorus (L.f.) Radlk.	Ulwathile, iLathile (X)	Bark, root , leaves	Heartwater and diarrhoea in cattle (Dold and Cocks, 2001; McGaw *et al.*, 2008), Diarrhoea and dysentery (Bisi-Johnson *et al.*, 2010)	Acute toxicity (Pendota *et al.*, 2010), anti inflammatory, analgesic antipyretic (Pendota *et al.*, 2009)	-
Scrophulariaceae					
Physalis peruviana L.	Igquzu (X)	Leaves	Stomach disorder (Bisi-Johnson *et al.*, 2010)	-	-
Physalis viscose L.	Umqumqumu (Z)	Leaves	Diarrhoea (Mlambo, 2008)	Antibacterial (Ovenden *et al.*, 2004)	Physaloside A (Ovenden *et al.*, 2004)
Jamesbrittenia atropurpurea (Benth.) Hilliard		Herb	Antispasmodic, stimulant; convulsions; cough; bronchitis (van Wyk, 2008)	-	-
Xanthium		Root	Cancer, dysentery, catarrh, leprosy (Watt	Anti-inflammatory and analgesic (Han	1-*O*-caffeoylquinic acid, 3-*O*-caffeoylquinic acid,

strumarium L.			and Breyer-Brandwijk, 1996; Fouche *et al.*, 2008)	*et al.*, 2007)	chlorogenic acid, 4-*O*-caffeoylquinic acid, cynarin, 1,4-*O*-dicaffeoylquinic acid, 1,5-*O*-dicaffeoylquinic acid, 1,5-*O*-dicaffeoylquinic acid, 1,3,5-*O*-tricaffeoylquinic acid, 3,4,5-*O*-tricaffeoylquinic acid (Han *et al.*, 2007)
Solanaceae					
Solanum aculeastrum Dun	umthuma (X, Z)	Fruit, root, leaves	Fruit decoction for haemorrhoids, dysentery, fruit as enema for diarrhoea (Bisi-Johnson *et al.*, 2010)	Antimicrobial (Koduru *et al.*, 2006); Anticancer (Koduru *et al.*, 2007)	tomatidine and solasodine (Koduru *et al.*, 2007)
Solanum incanum L.	uMthuma, *intfuma* (S)	Root	Root infusion for back ache (Amusan, 2007)	-	-
Solanum mauritianum	Umtotovane (Z)	Leaf	Infusion for dysentery and diarrhoea (Watt and Breyer-Brandwijk, 1962)	-	-
Solanum panduriforme E. Mey	Thuthula	Fruit sap	Diarrhoea (Van der Merwe *et al.*, 2001; McGaw *et al.*, 2008)	-	-
Solanum supinum Dun.	Thola (S)	Root	Decoction for diarrhoea (Mathabe *et al.*, 2006)	-	-
Sterculiaceae					
Withania somnifera (L.) Dun	uBuvimba		Fever, cold and flu, abdominal discomfort, diarrhoea, worms sedative and hypnotic (van Wyk and Gericke, 2000; Fouche *et al.*, 2008)	Anti-inflammatory, antitumor, immunomodulatory (Mishra et al., 2000); antichorelae (Acharya *et al.*, 2009)	Isopelletierine, anferine, withanolides, withaferins, sitoindosides (Mishra et al., 2000)
Dombeya rotundifolia (Hochst.) Planch.	Tshiluvhari (V)	Root, bark, wood	Internal ulcers, haemorrhoids, diarrhoea, stomach problems, nausea, chest pain (Verschaeve and Van Staden, 2008)	Mutagenicity , antimutagenicity	-
Hermannia incana Cav	Mavulakuvaliwe	leaves	Crushed with cold water and taken orally for diarrhoea (Appidi *et al.*, 2008)	Toxicological assay (Appidi *et al.*, 2009); antimicrobial, anti-inflammatory, antioxidant and cytotoxicity (Essop et al., 2008)	-
Waltheria indica L.		Whole plant	Decoction for diarrhoea (Mathabe *et al.*, 2006)	Antibacterial, antifungal and antiviral (Maregesi *et al.*, 2008)	-
Strychnaceae					
Strychnos madagascariensis Pior.	Umkwakwa, Mukwakwa (V)		Diarrhoea (de Wet *et al.*, 2010)	-	-
Ulticaceae					
Pouzolzia mixta solms	Muthanzwa	Root, leaves	Dysentery (Verschaeve and Van Staden, 2008); diarrhoea (Samie *et al.*, 2010)	-	-

Verbenaceae					
Clerodendrum glabrum E. Mey	Umqangazani Uqangazana (X), iNunkisiqaqa (X) Umqangazane	leaves	Bloody stool, chest infections (Bisi-Johnson *et al.*, 2010)	-	--
Viscaceae					
Viscum capense L. F.	Iphakama (Z)		Diarrhoea (Forbes, 1986; Van Wyk *et al.*, 2008)	-	-
Vitaceae					
Lippia javanica (Burm.f.) Spreng	Musudzungwane (V)	Leaf infusion	Prophylactics against dysentery, diarrhoea and malaria (Mabogo, 1990; Fouche *et al.*, 2008)	-	-
Rhoicissus tridentata (L.F.) Wild & Drums.	Umthwazi (Z), Murumbula-mbudzana (V)	Tuber decoction	Diarrhoea in goat and sheep (Dold and Cocks, 2001; McGaw *et al.*, 2008)	Antispasmolytic (Katsoulis *et al.*, 2000)	-
Cyphostemma cirrhosum (Thunb.)	Udekane (Z)	Leaves	Diarrhoea (Mlambo, 2008)	-	-
Zingiberaceae					
Aframomum latifolium (Afzel.) K. Schum		Leaves	Decoction for diarhhoea (De Villiers *et al.*, 2010)	-	-
Elytropappus rhinocerotis (L.f) Less.		Twigs	Bitter for dyspepsia, indigestion, diarrhoea (van Wyk, 2008)	-	-

V=Vhavenda, Z=Zulu, X=Xhosa, S=Swazi

Appendix 9.1: 1D and 2D NMR spectra data of Ursolic acid

Peak number	Hydrogen	13C/DEPT	HSQC	HMBC	LITERATURE
1	1.46-1.6	CH_2	37	15(C25), 27(C2), 56(C5), 78(C3)	39.2
2	1.38-1.5	CH_2	27	37(C1), 56(C5), 78(C3)	28.1
3	3.0	CH	78	16(C23), 27(C2)	78.2
4	-	C	39	-	39.6
5	0.64	CH	56	16(C23), 18 (C6), 37(C1)	55.9
6	1.26, 1.44	CH_2	18	56(C5)	18.8
7	1.24, 1.4	CH_2	33	56(C5)	33.7
8	-	C	39	-	40.1
9	1.42	CH	48	15(C25), 23(C11), 37 (C1), 38.8(C10), 39(C8)	48.1
10	-	C	37	-	37.5
11	1.76-1.88	CH_2	23	39 (C8), 125(C12), 140(C13)	23.7
12	5.2	CH	125	42(C14), 48(C9), 53(C18)	125.7
13	-	C	140	-	139.3
14	-	C	42	-	42.6
15	0.95, 1.4	CH_2	28	24(C16), 48(C17)	28.8
16	1.5, 1.9	CH_2	24	28 (C15), 42(C14), 48(C17), 53(C18), 178(C28)	25.0
17	-	C	48	-	48.1
18	2.08	CH	53	17.5(29), 24(16), 38.8(19), 42(14), 37(C20), 125(12), 140(13), 178(28)	53.6
19	0.9	CH	38.6	39(C19), 37(C20)	39.5
20	1.29	CH	37		39.4
21	1.48-1.58	CH_2	30		31.0
22	0.87, 1.48	CH_2	38.9	24(C16), 53(C18)	37.4
23	0.64	CH_3	16	29(C24), 39(C4), 56(C5), 78(C3)	16.5
24	0.87	CH_3	29	16(C23), 78(C3)	28.8
25	0.85	CH_3	15	37(C1), 56(C5)	15.7
26	0.72	CH_3	17.3	33(C7), 39(C8), 42(C14), 48(C9)	17.5
27	1.02	CH_3	23.4	28(C15), 39(C8), 42(C14), 140(C13)	24.0
28	-	C	178	-	179.7
29	0.78	CH_3	17.5	38.8(C19), 53(C18)	17.5
30	0.90	CH_3	22	31(C21), 48(C20)	21.4

Appendix 9.2: 1D and 2D NMR spectra data of mixture of corosolic acid and maslinic acid

Pea1k number	^{1}H	^{13}C/DEPT	HSQC (Corosolic acid)	HSQC (Maslinic acid)	HMBC (H→C)	LITERATURE
1	1.7-1.8, 0.73-0.79	CH_2	47.75	47.75	16.92(C25), 38.27(C10), 55.35(C5), 68(C2), 83.49(C3)	46.8
2	3.4	CH	68.41	68.41	83.49(C3)	68.9
3	2.7	CH	83.49	83.49	68.41(C2), 29.87(C24)	83.8
4	-	C	39.61	39.61	-	39.1
5	0.68-0.75	CH	55.35	55.35	39.61(C4), 38.25(C10), 29.43(C24)	55.4
6	1.41-1.47, 1.26-1.33	CH_2	18.71	18.71	38.12(C10), 39.61(C4)	18.4
7	1.36-1.45, 1.56-1.61	CH_2	33.00	33	17.49(C26), 55.35(C5), 18.71(C6)	32.9
8	-	C	39.75	39.75	-	39.6
9	1.5	CH	47.53	47.53	23.74 (C11) (24.01)	47.5
10	-	C	38.25	38.25	-	38.3
11	1.8-1.9, 1.43-1.47	CH_2	23.74	24.01	47.53(C9), 125.55(C12) (122.72), 139.43(C13) (145.09)	23.4
12	5.11	CH	125.55	122.72	23.74(C11) (24.01), 42.19(C14) (41.92), 47.53(C9), 53.05(C18) (41)	125.3
13	-	C	139.42	145.09	-	138.1
14	-	C	42.19	41.92	-	42.1
15	0.92-0.99, 1.75-1.79	CH_2	28.21	28.21	39.57(C8), 23.60(C27) (26.8), 42.19(C14) (41.92)	28.0
16	1.26-1.33, 1.47-1.52	CH_2	24.51	27.94	178 (C28) (179.24), 28.21(C15), 53.05(C18), (41), 48.13(C17)	24.3
17	-	C	48.33	48.33	-	48.1
18	2.09 (2.7)	CH	53.05	41.00	17.75(C29), 24.51(C16) (27.94), 39.10(C19) (27.94), 42.19(C14) (41.92), 48.13(C17), 37(C22),125.55(C12) (122.72), 139.42(C13) (145.09), 178 (C28) (179.24)	52.8
19	0.88-0.92	CH	39.07	27.94 (CH_2)		39.1
20	1.25-1.31	CH	39.02 (CH)	46.13 (C)		38.9
21	1.48-1.58	CH_2	30.60	37.00	24, 31	30.7
22	1.58, 1.00-1.09	CH	37.00	47.53	178(C28) (179.24) , 24.51(C16) (27.94)	36.7
23	0.90	CH_3	29.20	29.2	17.76(C24), 55.35(C5), 39.61(C4)	28.7
24	0.68	CH_3	17.76	17.76	29.43(C23), 39.61(C4), 55.35(C5)	17.0
25	0.88	CH_3	16.94	16.94	38.25(C10), 47.53(C9)	17.0
26	0.72	CH_3	17.40	17.40	47.53(C9), 42.19(C14) (41.92), 33.01(C7)	17.0
27	1.00	CH_3	23.60	26.80	28.21(C15), 39.75(C8), 42.19(C14) (41.92), 139.42(C13) (145.09)	23.7
28	-	C	178.00	179.70	-	177.9
29	0.79	CH_3	17.50	17.50	39.07(C19) (27.94), 53.05(C18) (41)	17.0
30	0.88	CH_3	21.60	21.60	39.02(C20) (46.13)	21.2

Appendix 9.3: 1D and 2D NMR spectra data of mixture of asiatic aicd and arjunolic acid

Peak number	^{1}H	^{13}C/DEPT	HSQC (Asiatic acid)	HSQC (Arjunolic acid)	HMBC (H→C)	LITERATURE
1	0.69, 1.73	CH_2	48.31	48.31	18.04(C25), 68.67(C2), 76.50(C3)	46.8
2	3.45	CH	68.67	68.67	76.50(C3)	68.9
3	3.13	CH	76.50	76.50	68.67(C2), 14.30(C24), 65.01(C23), 43.62(5)	83.8
4	-	C	43.62	43.62	-	39.1
5	1.14	CH	47.27	47.27	76.50(C3), 47.27(C4), 65.01 (C23), 14.30(C24), 33.40 (C7), 18.04 (C25)	55.4
6	1.33, 1.18	CH_2	18.03	18.03	38.12(C10), 39.61(C4)	18.4
7	1.44, 1.20	CH_2	33.40	33.40	40.49 (C8)	32.9
8	-	C	40.49	40.49	-	39.6
9	1.5	CH	47.51	47.54	23.74 (11) (24.01)	47.5
10	-	C	38.40	38.40	-	38.3
11	1.78, 1.42	CH_2	23.00	24.01	125.19(C12) (122.72), 138.92(C13) (145.09)	23.4
12	5.09 (5.12)	CH	125.19	122.28	42.21(C14) (41.92), 47.51(C9) (47.54), 52.62(C18) (41.73)	125.3
13	-	C	138.71	144.68	-	138.1
14	-	C	42.21	41.92	-	42.1
15	1.73, 1.58	CH_2	28.07	28.21	39.57(C8), 23.60(C27) (26.8), 42.19(C14) (41.92)	28.0
16	1.88, 1.48	CH_2	24.31	27.94	179.13 (C28) (179.24),	24.3
17	-	C	47.99	48.33	-	48.1
18	2.05 (2.72)	CH	52.62	41.73	18.16(C29), 24.31(C16) 39.44(C19) (27.94), 42.21 (C14) (42.52), 47.99(C17) (47.01), 125.19(C12) (122.28), 138.92(C13) (144.68), 179.13 (C28) (179.24)	52.8
19	0.88-0.92	CH	39.44	24.31(CH_2)		39.1
20	1.25-1.31	CH	38.90(CH)	47.27 (C)		38.9
21	1.38, 1.20	CH_2	31.42	31	24, 31	30.7
22	1.55. 1.39	CH_2	33	33	24.31(C16), 31.61 (C21)	36.7
23	3.25, 3.00	CH_2	65.01	65.01	14.91(C24), 43.62(C5), 47.27(C4), 76.50 (C3)	28.7
24	0.50	CH_3	14.91	14.91	65.01(C23), 47.27(C4), 43.62(C5)	17.0
25	0.65	CH_3	18.04	18.04	40.49(C10), 43.62(C5), 48.31(C1)	17.0
26	0.68	CH_3	17.04	17.04	47.53(C9), 42.19(C14) (41.92), 33.18 (C7)	17.0
27	0.99 (1.04)	CH_3	23.40	26.80	28.48(C15), 40.49(C8) (39.96), 42.21(C14) (42.57), 138.92(C13) (144.68)	23.7
28	-	C	179.13	179.24	-	177.9
29	0.77 (0.82)	CH_3	18.16	26.0	39.44(C19) (27.94), 53.05(C18) (41)	17.0
30	0.87 (0.82)	CH_3	23.00	33.000	39.02(C20) (46.13)	21.2

Appendix 9.4: 1D and 2D NMR spectra data of combretastatin B5-2'-O- glucopyranoside

Peak number	^1H	^{13}C/DEPT	HSQC[a]	HMBC (H→C)	LITERATURE DATA HSQC[a']
1		C	132.72		132.43
2	6.5	CH	106.26	C1, C3, C6, C1a	105.96
3		C	144.35		144.77
4		C	133.79		133.44
5		C	148.25		147.77
6	6.5	CH	106.26	C1, C5, C2, C1a	105.96
1a	2.7	CH_2	36.96	C1, C2, C1a', C1'	36.58
1a'	2.9, 3.0	CH_2	31.73	C1, C1', C2', C6', C1a	31.35
1'		C	128.55		128.22
2'		C	144.35		143.95
3'		C	139.71		139.29
4'		c	147.26		146.90
5'	6.7	CH	109.40	C1', C3', C4', C6'	109.10
6'	6.6	CH	118.95	C2', C4', C1a'	118.68
1"	4.5	CH_2	106.16	C2', C2", C5",	105.79
2"	3.3	CH	74.45	C1", C3"	74.04
3"	3.2	CH	76.62	C4", C5"	76.14
4"	3.2	C	70.15	C3", C5"	69.71
5"	3.2	CH	77.85	C3"	77.44
6'	3.5, 3.7	CH_2	61.34	C4", C5"	60.89
3-OCH_3	3.5	CH_3	56.34	C3	56.00
4-OCH_3	-	CH_3	-		-
5-OCH_3	3.5	CH_3	56.34	C5	56.00
4'-OCH_3	3.5	CH_3	56.30	C4'	56.00

b Pelizzoni Francesca, 1994: Combretastatin derivatives with antitumoral activity and process for the preparation thereof. Patent Cooperation Treaty (PCT), WO 94/05682, CO7H 15/203, CO7C 43/23, A61K 31/70, 31/085

Appendix 9.5: 1D and 2D NMR spectra data of combretastatin B1-2'-O- glucopyranoside

Peak number	^1H	^{13}C/DEPT	HSQC[b]	HMBC (H→C)	LITERATURE DATA HSQC[b']
1		C	138.54		138.10
2	6.5	CH	105.60	C1, C1a, C3, C4	105.63
3		C	152.69		152.65
4		C	135.53		135.45
5		C	152.72		152.65
6	6.5	CH	105.60	C1, C1a, C4, C5	105.63
1a	2.69, 2.79	CH_2	36.92	C1, C1', C2, C6, C1a'	36.91
1a'	2.92, 3.20	CH_2	31.34	C1a, C1', C2', C6'	31.18
1'		C	128.27		128.03
2'		C	143.78		143.93
3'		C	139.13		139.35
4'		c	147.02		146.95
5'	6.7	CH	108.54	C1', C3', C4',	108.99
6'	6.6	CH	119.24	C1a', C2', C4'	118.56
1"	4.5	CH_2	105.69		105.77
2"	3.3	CH	74.21		74.09
3"		CH	76.63		76.26
4"		C	69.72		69.76
5"	3.2	CH	77.03		77.51
6'	3.5, 3.7	CH_2	60.97		60.96
3-OCH_3	3.5	CH_3	55.14	C3	55.78
4-OCH_3	3.5	CH_3	59.69	C4	60.04
5-OCH_3	3.5	CH_3	55.34	C5	55.78
4'-OCH_3	3.5	CH_3	55.42	C4'	55.94

b Pelizzoni Francesca, 1994: Combretastatin derivatives with antitumoral activity and process for the preparation thereof. Patent Cooperation Treaty (PCT), WO 94/05682, CO7H 15/203, CO7C 43/23, A61K 31/70, 31/085

Appendix 9.6: 1D and 2D NMR spectra data of 3β-ethyl sitosterol

Peak number	^1H	^{13}C/DEPT	HSQC	HMBC (H→C)	LITERATURE
1	1.036, 1.817	CH_2	37.47		37.3
2	2.233	CH_2	31.8	36.73 (C10), 72.04 (C3), 121.93 (C6), 140.97 (C5)	31.9
3	3.502	CH	72.04		71.8
4		CH_2	40.00		40.5
5	-	C	140.97		140.7
6	5.33	CH	121.93	32.12 (C7), 36.73 (C10)	121.7
7	1.427	CH_2	32.12		31.9
8		CH	29.91		31.6
9		CH	50.35		50.2
10	-	C	36.73		36.5
11		CH_2	21.30		21.1
12	2.194	CH_2	40.00		39.8
13	-	C	42.54		42.3
14	1.058	CH	56.99		56.8
15	0.988	CH_2	24.52		24.3
16	1.234	CH_2	28.47		28.3
17	1.058	CH	56.27		56.1
18	0.988	CH_3	12.08	140.97 (C5), 37.47 (C1), 50.35 (C9)	11.9
19	0.659	CH_3	20.03	56.99 (C14), 56.23 (C17), 42.52 (C12), 42.54 (C13),	19.5
20	1.326	CH	36.36		36.2
21	0.904	CH_3	19.24	56.27 (C17), 36.36 (C20), 34.16 (C22)	18.9
22		CH_2	34.16		33.9
23		CH_2	26.29		26.1
24		CH	46.05		45.8
25		CH	29.37		29.1
26	0.988	CH_3	19.61		19.4
27	0.796	CH_3	19.00	29.37 (C25), 46.05 (C24), 19.61 (C26)	19.1
28		CH_2	23.28		23.1
29	0.822	CH_3	12.20	23.25 (C28)	12.0
$\underline{CH_3}CH_2$		CH_3			
$CH_3\underline{CH_2}$		CH_2	76.86		

Appendix 9.7: 1D and 2D NMR spectra data of Quercetin

Peak number	^1H	^{13}C/DEPT	HSQC	HMBC (H→C)	LITERATURE
2		C	147.96	-	146.8
3		C	137.23	-	135.8
4		C	177.31	-	175.9
5		C	162.50	-	160.8
6	6.17	CH	99.28	162.50 (C5), 104.51 (C10), 94.39 (C8)	98.2
7		C	165.55	-	163.9
8	6.37	CH	94.39	165.55 (C7), 158.21 (C9), 104.51 (10), 99.28 (C6)	93.4
9		C	158.21	-	156.2
10		C	104.51	-	1103.0
1'		C	124.13	-	122.0
2'	7.87	CH	116.30	147.94 (C2), 144 (C3'), 121 (C6')	115.1
3'		C	147.96	-	145.1
4'		C	148.75	-	147.7
5'	6.88	CH	116.78	144 (C3'), 121 (C6')	115.6
6'	7.72	CH	121.65		120.6

Appendix 9.8: 1D and 2D NMR spectra data of Myricetin

Peak number	^{1}H	^{13}C/DEPT	HSQC	HMBC (H→C)	LITERATURE
2		C	146.57	-	148.2
3		C	135.92	-	137.5
4		C	175.86	-	177.5
5		C	161.05	-	162.6
6	6.17	CH	97.80	164.17 (C7), 161.05 (C5), 103.06 (C10), 99 (C6)	99.5
7		C	164.17	-	165.8
8	6.37	CH	92.95	164.17 (C7), 158 (C9), 103.06 (C10), 99 (C6)	94.6
9		C	156.76	-	158.4
10		C	103.06	-	104.7
1'		C	121.65	-	123.3
2'	7.38	CH	107.10	145.29 (C3'), 135.51 (C4'), 121.65 (C1'), 107.10 (C6')	108.8
3'		C	145.29	-	146.9
4'		C	135.51	-	137.1
5'		C	145.29	-	146.9
6'	7.38	CH	107.10	145.29 (C5'), 135.51 (C4'), 121.65 (C1'), 107.10 (C2')	108.8

Appendix 9.9: 1D and 2D NMR spectra data of Isoetin 2' methyl ether/ Isoetin 4' methyl ether

Peak number	^{1}H	^{13}C/DEPT		HMBC (H→C)	LITERATURE	
2	-	C	163.09	C3, C6'	163.2[a]	161.70[b]
3	7.2(s)	CH	108.85	C6'	108.7	106.77
4	-	C	184.24		183.5	181.74
5	-	C	163.67		162.7	161.41
6	6.2(d, J=)	CH	99.97		99.8	98.48
7	-	C	166.22		165.3	163.88
8	6.4 (d, J=)	CH	94.86	C6	95.1	93.48
9	-	C	159.43		159	157.19
10	-	C	105.09	C3, C8	105.1	103.46
1'	-	C	110.17	C3'	108.9	106.97
2'	-	C	153.27	OCH_3, C3', C6'	154.2	150.50
3'	6.65 (s)	CH	101.36		105.4	104.20
4'	-	C	153.27	OCH_3, C3', C6'	152.8	151.60
5'	-	C	140.86	C3', C6'	142.8	138.73
6'	7.38 (s)	CH	114.44		112.6	113.44
OCH_3	3.8 (s)		56.34 at C2' or C4'		57.6 at C5'	-

[a]Isoetin 5'methyl ester (AbdurRahman and Moon, 2007), [b] isoetin (Gluchoff-Fiasson *et al.*, 1991)

Appendix 9.10: 1D and 2D NMR spectra data of Quercetin-3-O-β-galactopyranoside

Peak number	^1H	^{13}C/DEPT	HSQC	HMBC (H→C)	LITERATURE
2	-	C	156.76	-	158.3
3	-	C	133.85	-	135.8
4	-	C	177.83	-	179.4
5	-	C	161.42	-	163.0
6		CH	99.17	162.50 (C5), 104.51 (C10), 94.39 (C8)	99.8
7	-	C	164.58	-	166.0
8		CH	94.09	165.55 (C7), 158.21 (C9), 104.51 (10), 99.28 (C6)	94.7
9	-	C	156.80	-	158.8
10	-	C	102.36	-	104.2
1'	-	C	122.14	-	123.2
2'		CH	115.66	147.94 (C2), 144 (C3'), 121 (C6')	117.8
3'	-	C	144.97	-	145.8
4'	-	C	148.75	-	149.9
5'		CH	116.47		116.1
6'		CH	121.42		122.9
1''	5.18 (d)	CH	104.24	133.85(C2)	105.4
2''	3.83 (t)	CH	71.55	104.12 (C1''), 73.70 (C3'')	73.2
3''	3.56 (m)	CH	73.40	104.12 (C1''), 71.87 (C2''),	75.1
4''	3.87 (s)	CH	68.30	144 (C3'), 121 (C6')	70.0
5''	3.94 (t)	CH	75.96		77.2
6''	3.66 (dd), 3.5 (m)	CH_2	60.45	75.80 (5''), 68.61 (C4'')	61.9

Appendix 9.11: 1D and 2D NMR spectra data of Myricetin-3-O-β-galactopyranoside

Peak number	^1H	^{13}C/DEPT	HSQC	HMBC (H→C)	LITERATURE
2		C	156.95	-	156.4
3		C	134.56	-	135.4
4		C	177.97	-	177.6
5		C	161.54	-	161.4
6	6.19	CH	98.47	164.70 (C7), 161.54 (C5), 104.12 (C10), 93.25 (C8)	98.7
7		C	164.70	-	164.4
8	6.38	CH	93.25	164.70 (C7),157.24(C9), 104.12 (C10), 98.47 (C6)	93.4
9		C	157.24	-	156.4
10		C	104.14	-	103.9
1'		C	120.26	-	120.0
2'	7.37	CH	108.52	156.95 (C2), 144.95 (C3'), 136.71 (C4'), 120.26 (C1'), 108.52 (C6')	108.6
3'		C	144.95	-	145.5
4'		C	136.71	-	136.8
5'		C	144.95	-	145.5
6'	7.37	CH	108.52	156.95 (C2), 144.95 (C5'), 136.71 (C4'), 120.26 (C1'), 108.52 (2')	108.6
1''	5.18 (d)	CH	104.12	134.56 (C2)	105.4
2''	3.83 (t)	CH	71.87	104.12 (C1''), 73.70 (C3'')	73.2
3''	3.56 (m)	CH	73.70	104.12 (C1''), 71.87 (C2''),	75.1
4''	3.87 (s)	CH	68.61		70.0
5''	3.94 (t)	CH	75.80		77.2
6''	3.66 (dd), 3.5 (m)	CH_2	60.52	75.80 (5''), 68.61 (C4'')	61.9