Polarity of extracts and fractions of four *Combretum* (Combretaceae) species used to treat infections and gastrointestinal disorders in southern African traditional medicine has a major effect on different relevant *in vitro* activities

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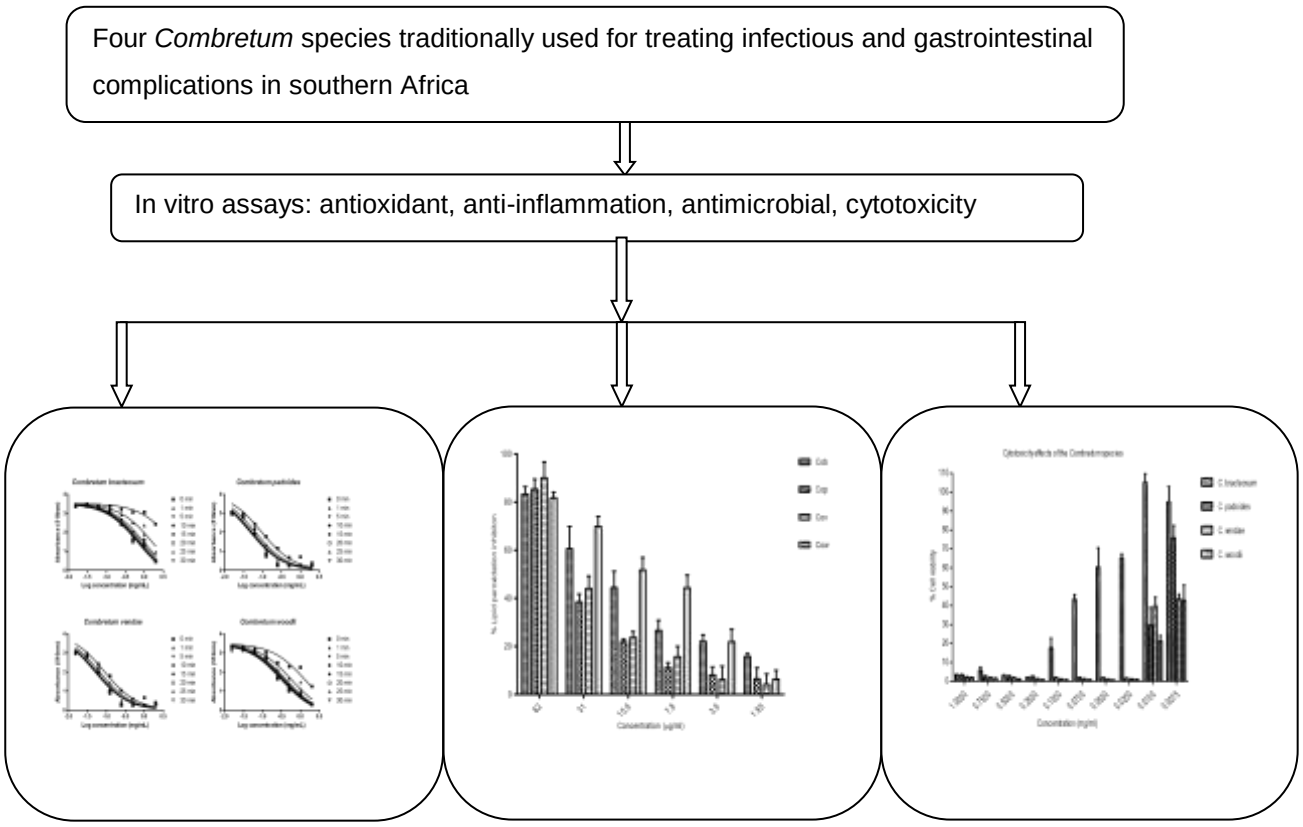
Graphical abstract

Phenolic-enriched extracts of four *Combretum* species namely *Combretum bracteosum*, *Combretum padoides*, *Combretum vendae* and *Combretum woodii* used in South African traditional medicine to treat various ranges diseases were evaluated against some diarrhoeal aetiologies. All the crude extracts and their fractions of varied polarities had good antimicrobial (non-polar fractions) or good antioxidant (polar fractions) activity. The crude extract of all the plant species were cytotoxic to Vero cell lines but did not lead to COX enzyme inhibition.



Abstract

Ethnopharmacological importance: Infections and gastrointestinal (GIT) disorders such as diarrhoea causes many problems in human health and animal production. Many *Combretum* species are used in traditional medicine to treat various diseases by rural people in Africa and Asia. Much of the work done to date on some species to validate their ethnopharmacological use was on the non-polar or intermediate polarity components. Many species are yet to be studied against relevant disease parameters using more polar extracts.

Aims: The polar components were extracted and fractionated by solvent-solvent fractionation to yield fractions of different polarities. The activity of these fractions on different parameters that could be involved in infectious and gastrointestinal track (GIT) disorders was investigated. The cytotoxic activities of the extracts were also determined to evaluate the potential of these extracts to combat diarrhoea in production animals.

Materials and Methods: Phenolic-enriched leaf extracts of *Combretum bracteosum* (Cob)*, Combretum padoides* (Cop)*, Combretum vendae* (Cov) and *Combretum woodii* (Cow) were obtained by extracting with a mixture of 70% acetone acidified with 1% HCl and n-hexane. The extract was sequentially treated by solvent-solvent fractionation with dichloromethane, ethyl acetate, and butanol to yield fractions with a large variation in polarity. The phenolic constituents of the extracts and fractions were determined using standard procedures. The antioxidant activities were determined using various standard methods. The minimum inhibitory concentrations (MICs) of the crude extracts and fractions against four bacterial and three fungal strains were assessed with a microplate serial dilution method. Cyclooxygenase (COX) and lipoxygenase (LOX) enzyme inhibitory assays and cytotoxicity studies against Vero cells were also carried out.

Result: Some of the fractions had much higher antioxidant activity than the positive controls. The average EC_{50} values of the extracts for the DPPH and ABTS antioxidant assays were 0.21-12 µg/ml (Cop), 0.25-16 µg/ml (Cov), 0.33-9.41 µg/ml (Cow) and 4.97-85 µg/ml (Cob) respectively while the mean EC_{50} values for the positive controls ascorbic acid and trolox were 1.28-1.51 and 1.02-1.19 µg/ml respectively. All the crude extracts inhibited lipid peroxidation of linoleic acid by more than 80% at a concentration of 64 µg/ml.

Even though some crude extracts had relatively low antimicrobial activity, fractions from these had high activity. Cop had the highest antibacterial activity with MICs ranging between 19-2500 µg/ml,

followed by Cov with MICs ranging between 39-625 µg/ml. Cop also had the highest antifungal activity with MICs between 19-625 µg/ml. The MIC for Cow and Cov ranged from 19 to 1250 µg/ml. The extracts had no activity against COX 1 and 2 enzymes in the anti-inflammatory assay but had good lipoxygenase inhibition. The crude extracts had high concentrations of hydrolysable tannin (gallotannin). A good correlation (R^2= 0.99) was found between the antioxidant activity and total tannin content indicating that, gallotannins may be responsible for the antioxidant activity.

Conclusion: The results obtained in this study provided a scientific basis for the use of leaf extracts from these plant species to treatinfectious and GIT disorders. In general non-polar fractions had a high antimicrobial activity and polar fractions had a high antioxidant activity.

Keywords: *Combretum*; infection; gastrointestinal disorders; diarrhoea; intestinal inflammation; cytotoxicity

Abbreviations

$ABTS^+$ = 2, 2'-azino-bis (3-ethylbenzothiazoline)-6-sulphonic acid radical; ac = acetone; $AlCl_3$ = aluminium chloride; ANOVA = analysis of variance; ATCC = American type culture collection; CE/g = catechin equivalent per gram; COX = cyclooxygenase; CT = condensed tannin; cyn-3-glu = cyanidin-3-glucose; DCM = dichloromethane; DF = dilution factor; DMSO= dimethyl sulphoxide; DPPH = 2, 2'-diphenyl-1-picrylhydrazyl; EC_{50} = dose needed to scavenge free radical by 50%; ENS = enteric nervous system; FRAP = ferric reducing antioxidant power; $GAEg^{-1}$ = gallic acid equivalent in mg per g; GIT = gastrointestinal tract; INT = p-iodonitrotetrazolium violet; l = path length in cm; LC_{50} = dose needed to induce cytotoxic effect on Vero cells by 50%; LOX = lipoxygenase; LTs = leukotrienes; MDA = malondialdehyde; MEM = minimal essential medium; MHA = Mueller Hinton agar; MICs = minimum inhibitory concentrations; MTT = [3-(4, 5-dimethylthiazol-2-yl)-2, 5 diphenyl tetrazolium bromide]; MW = molecular weight; PBS = phosphate buffer solution; PCI = prostacyclin; PGE = prostaglandin E; PVPP = polyvinylpolypyrrolidine; QE/g = quercetin equivalent/g; R_f = retardation factor; RH = relative humidity; SDA = Sabouraud dextrose agar; SOD = superoxide dismutase.

1.0 Introduction

Effective treatment of opportunistic infection and gastrointestinal track (GIT) disorders caused by wide range of enteropathogenic organisms is still a major challenge in healthcare delivery. This problem is compounded by increasing cases of immunocompromised patients due to human immunodeficiency virus (HIV) infection particularly in the southern Africa region (UNAIDS, 2008), despite developments in mainstream medicine. Pathogenic microbe infections act via multiple approaches such as intoxication (release of toxins), food poisoning, invasion of body tissues (adhesion and colonisation) and modulation of immune defence system. Systematic infections normally cause fevers, chills, sweats, malaise, headache, muscle and joint pain, or changes in mental status while localized infections produce tissue inflammation (redness, swelling, tenderness and heat) and loss of function.

Some of the commonly encountered opportunistic and enteropathogenic microbes include *Escherichia coli*, a Gram-negative bacterium present in normal human and animal flora. However, the pathogenic strains are implicated in causing serious diseases or symptoms such as diarrhoea, haemorrhagic colitis, haemolytic uremic syndrome and thrombocytopenic purpura (Bell and Kyriakides, 1998). *Pseudomonas aeruginosa* is a Gram-negative opportunistic highly antibiotic resistant bacterial pathogen responsible for infections in the urinary tract, respiratory system, soft tissue, bone and joint, gastrointestinal and a variety of systemic infections, dermatitis and bacteremia particularly in patients with severe burns, cancer and AIDS patients (Andualem, 2012). *Staphylococcus aureus*, a Gram-positive bacterium, causes mastitis, toxic shock syndrome (TSS) and staphylococcal food-poisoning (SFP) in human and animal. SFP symptoms caused by ingestion of food containing heat-stable staphylococcal enterotoxins (SETs) include nausea, vomiting, abdominal cramps and diarrhoea (Rosengren *et al*., 2010). The microbe has also been implicated in causing skin infection such as boils, abscesses, carbuncles and sepsis of wounds. *Enterococcus faecalis* is another Gram-positive bacterium and natural inhabitant of the gastrointestinal tract of humans and is usually disseminated from the gastrointestinal tract to cause cholangitis, peritonitis, and intra-abdominal abscess. Enterococci are a leading cause of nosocomial infection and an infrequent cause of pneumonia, meningitis and osteomyelitis, usually in the immunocompromised host (Butler, 2006).

Candida albicans is one of the opportunistic fungi that can infect the mouth, skin, intestine or vagina. Rapid increases in diseases associated with fungal infection such as candidiasis are related to the increased rate of HIV infection (Shai *et al*., 2008). *Cryptococcus neoformans* is an environmental opportunistic pathogenic fungus, responsible for serious infections in immunocompromised patients. It causes diseases in any organ of the human body following dissemination from lungs to other organs through blood circulation. These diseases include devastating meningoencephalitis of the central nervous system, cerebellar cryptococcoma, pneumonia and colonic cryptococcosis (Vecchiarelli and Monari, 2012). *Aspergillus fumigatus* is the most prevalent airborne saprophytic primary and opportunistic fungal pathogen as well as a major allergen. The organism causes life-threatening pulmonary or fatal invasive aspergillosis in immunocompromised hosts (Nierman *et al.*, 2005).

Antibiotics are effective in combating many infectious diseases, but improper uses enhance selection of resistant microbes making them less susceptible to conventional drugs (Barbour *et al*.,

2004). Medicinal plants are used worldwide to supply basic healthcare needs and to combat many kinds of diseases. The therapeutic properties of plants are attributed to the presence of complex mixtures of phytochemicals acting individually or synergistically. Some of the plant compounds may be novel bioactive substances that can be effective as therapeutic agents for treating ailments with a mechanism different from currently available drugs. Extracts of many medicinal plants have been investigated for antimicrobial activity(Maregesi *et al*., 2008) and many have microbicidal or microbistatic activity.

Infection, malabsorption, and malnutrition are potent oxidizing stimuli leading to increased concentrations of reactive oxygen species (ROS) or reactive nitrogen species (RNS) and depleted endogenous antioxidant defences. In innate immune system, macrophages and other phagocytes engulf invading pathogens and fuse the pathogen-containing phagosome with a lysosome containing lysosomal enzymes that destroys the pathogen. This process is aided by the generation of hydrogen peroxide, superoxide and hydroxyl radicals (Woodsford *et al*., 2010). Imbalance between production and destruction of ROS/RNS creates a homeostatic distortion resulting in oxidative stress (Catala, 2009). Oxidative stress has been implicated in aetiology of many infectious and non-infectious diseases (Aruoma, 1996). Series of endogenous antioxidant defence systems moderate the amount of ROS/RNS in the body while other antiradicals are derived from exogenous sources such as diet and medicinal plants. Plants with high antioxidant activity may prevent or alleviate diseases associated with oxidative stress (Halliwell, 2008).

Infection and oxidative stress also induces the activation of inflammatory mediators (Valko *et al*., 2007). Although inflammation is an indispensable defence mechanism adapted by the body, excessive and prolonged inflammatory response can be detrimental (Ames *et al*., 1993). Inflammatory responses are controlled by a number of chemical mediators such as prostanoids (prostaglandin E (PGEs), prostacyclin (PCI) and leukotrienes (LTs)). These prostanoids play central roles in the inflammatory response to infections and are involved directly in diarrhoea as secretagogues and enteric nervous system (ENS) stimulant. Modulating rate-limiting enzymes involved in the biosynthesis of prostanoids such as cyclooxygenase (COX-1 and COX-2) and lipoxygenases (LOXs) can control inflammation. Many medicinal plant extracts have been investigated for anti-inflammatory activities (Iwalewa *et al*., 2007) and some modulate excessive production of COX enzymes.

Antioxidant molecules can also serve as possible therapeutic agents in inflammatory conditions, in cases where they mediate and modulate the activation of inflammatory genes (Valko *et al*., 2007).

Combretum is the largest genus of the Combretaceae family with about 370 species (McGaw *et al*., 2001). Several species *of Combretum* are used in traditional medicine for treating various kinds of diseases by many communities across Africa (Eloff, *et al*., 2008). Phytochemical studies of a number of *Combretum* species have led to isolation of triterpenoids, stilbenoids and flavonoids (Eloff, *et al*., 2008) with different pharmacological activities such as antimicrobial (Eloff, 1999), anti-inflammatory (Angeh *et al*., 2007), anticarcinogenic (Pettit *et al*., 1988), and antioxidative activity (Aderogba *et al*., 2011).

To validate the ethnomedicinal use of some *Combretum* and their closely related species used for treating infectious and GIT disorders, we used a solvent system different from conventional non-polar that would break cellular membranes and extract highly polar compounds as well as non-

Table 1
Ethnobotanical information on some South African *Combretum* species used in treating infectious and gastrointestinal disorders

Plant species	Part use	Ethnopharmacological information	Biological activities investigated	Bioactive compound(s) isolated
C. bracteosum (Hochst.) Brandis ex Engl. (Xhosa: uqota, uqoto (Dold and Cocks, 1999))	Leaves and root	Epilepsy and convulsions (Risa *et al*., 2004)	$GABA_A$-benzodiazepine receptor binding assay (Risa *et al*., 2004); anti-inflammatory, anthelminthic and antischistosomal (McGaw *et al*., 2001)	
C. padoides Engl. & Diels	Leaves and root	Bloody diarrhoea, wounds and conjunctivitis (Gathirwa *et al*., 2011); antimalaria (Gathirwa *et al*., 2011; Nguta *et al*., 2010)	anti-inflammatory, anthelminthic 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)
C. vendae A.E. van Wyk (Afrikaans: Vendaboswilg. English: Venda bushwillow. Venda: Gopo-gopo (Hahn, 2012))	Leaves	Leprosy, ophthalmic remedy, and blood purification (Watt and Breyer-Brandwijk, 1962; Suleiman *et al*., 2010)	Antimicrobial (Suleiman *et al*., 2010)	apigenin (Eloff *et al*., 2008)
C. woodii Dümmer	Leaves	*C. woodii* is used interchangeably with *C. erythrophyllum* (Eloff *et al*., 2005a) for treating abdominal pains and venereal diseases (Hutchings *et al*., 1996)	anti-inflammatory, anthelminthic and antischistosomal (McGaw *et al*., 2001); antifungal (Masoko *et al*., 2007); antibacterial (Eloff *et al*., 2005a and b); anti coccidiosis (Naidoo *et al*., 2008)	2',3',4-trihydroxyl-3,5,4'-trimethoxyl bibenzyl (combretastatin B5) (Eloff *et al*., 2005b)

polar compounds. We then determined the antioxidant, antimicrobial, anti-inflammatory activities of phenolic-enriched crude extracts and fractions of varying polarity of four *Combretum* species indigenous to South Africa (Table 1). The phenolic compositions and cytotoxicity of the leaf crude extracts were also determined. The plant species were: *Combretum bracteosum* (Hochst.) Brandis ex Engl., *Combretum padoides* Engl. & Diels, *Combretum vendae* A. E. van Wyk and *Combretum woodii* Dümmer).

2.0 Material and Methods

2.1. Plant collection and treatment

The *Combretum* species were selected based on information from previous work in the Phytomedicine Programme (www.up.ac.za/phyto) and a literature survey on their uses in traditional medicine across Africa in treating gastrointestinal complications including diarrhoea. The leaves of *C. bracteosum*, *C. padoides*, *C. vendae* and *C. woodii* were collected from the Manie van der Schijff Botanical Garden of the University of Pretoria. Voucher specimens of the plants (*C. bracteosum* (PRU 117443), *C. padoides* (PRU 115416), *C. vendae* (PRU 50800) and *C. woodii* (PRU 20544)) were stored in the H G WJ Schweickerdt Herbarium of the Department of Plant Science, University of Pretoria. The leaf materials were dried at room temperature under shade for 14 days and ground to a powder.

2.2. Preparation of extracts

Phenolic-enriched extracts of the dried pulverised leaves (20 g) were prepared by simultaneous extraction and fractionation with a mixture of acidified acetone water (140 ml acetone, 60 ml water and 0.2 ml HCl (Naczk and Shahidi, 2004) and n-hexane (200 ml) for 24 h to obtain two phases. The lower phase of phenolic-enriched fraction referred to as the crude extract was separated from the upper phase of terpenoid-enriched n-hexane fraction The upper phase was dried under a stream of air at room temperature. The phenolic-enriched fraction was concentrated by evaporation to remove acetone at 40^{o}C under reduced pressure using a rotary evaporator. The resultant water soluble mixture was divided into two portions (A and B). Portion A was freeze dried (crude) while portion B was sequentially fractionated by solvent-solvent fractionation to yield the dichloromethane (DCM), ethyl acetate (ET), butanol (BU) and residual water (W) fractions respectively. The ET and BU

fractions were dried and further subdivided by extracting the dried powdered extract with acetone and methanol to obtain an acetone soluble fraction of ET (ET ac), BU (BU ac) and methanol soluble fraction of ET (ET met) and BU (BU ac). Solutions of 10 mg/ml of the crude extracts and fractions were prepared in acetone, 70% acetone in water or DMSO where relevant for phytochemical and pharmacological assays.

2.3. Crude extracts phenolic composition

2.3.1. Total phenolic content of the crude extract

Folin-Ciocalteau phenol reagent was used to determine total phenolic constituents of the crude extracts as described by Makkar (2003). The absorbance of plant extracts and a prepared blank were measured at 765 nm using a spectrophotometer.

Using the same 0.1% HCl acidified 70% acetone extract, polyvinylpolypyrrolidine (PVPP) binding method was used for evaluating the total tannin and non-tannin contents as described by Makkar (2003) .The total phenolic content, non-tannin and total tannin were determined from the standard curve of gallic acid expressed as gallic acid equivalent in mg per g (mg $GAEg^{-1}$) dried plant material using the following linear equation based on the calibration curve:

$$A = 4.9022TP \text{ (mg GAE.g}^{-1}\text{)}, (R^2=0.98).$$

where A is the absorbance and TP is the amount of phenolic, non-tannin and/or total tannin (mg GAE g^{-1} DW).

2.3.2. Determination of condensed tannin

The condensed tannin content of the crude extracts was determined using the vanillin/HCl assay described by Heimler *et al* (2006). An appropriate standard curve was prepared using catechin withconcentrations ranging from 0.0019 to 0.25 mg/ml.The amount of condensed tannin in the extracts expressed as catechin equivalent (CE)/g dried plant material was calculated from the equation:

$$A = 0.1791 \times (CT) \text{ mg CE g}^{-1} + 5.04 \times 10^{-2}, R^2=0.94$$

where A is the absorbance of the extracts in presence of an indicator and CT is the condensed tannin content.

2.3.3. Determination of proanthocyanidin

The proanthocyanidin content of the crude extracts was determined using the butanol-HCl assay described by Makkar (2003). Condensed tannin (% of dry matter) was calculated as equivalent amount of leucocyanidins using the following formula.

$$\text{Proanthocyanidin} = \frac{A_{550\ nm} \times 78.26 \times \text{dilution factor}}{\%\ \text{dry matter}}$$

where $A_{550\ nm}$ is the absorbance of the sample at 550 nm and 78.26 is the molecular weight of leucocyanidin. The dilution factor is equal to 1 without dilution solution (50% methanol) to prevent the reading to exceed 0.6 absorbance values. Where 50% methanol was added to prevent the absorbance values exceeding 0.6, the dilution factor is 0.5 ml/volume of extract taken.

2.3.4. Determination of hydrolysable tannin (gallotannin)

The potassium iodate assay was used to determine the gallotannin content of the crude extracts as described by Vermerris and Nicholson (2006). A standard curve was prepared using gallic acid under the same conditions as the extracts and results were expressed as gallic acid equivalent (GAE)/g dried plant material.

2.3.5. Determination of total flavonoids and flavonol

The total flavonoid content of the crude extracts was determined by the aluminium chloride method as described by Abdel-Hameed (2009). A standard curve was prepared using quercetin (0.0019-0.25 mg/ml) in methanol under the same conditions. The flavonoid concentration was expressed as mg quercetin equivalent/g of dry plant material (mg QE/g) derived from the following equation based on the calibration curve:

$$A = 4.9747 \times TF\ (\text{mg QE g}^{-1}),\ R^2 = 0.9846$$

where A is the absorbance at 420 nm and y is the concentration (mg QE g^{-1} dry plant material).

The flavonol content of the extracts was also determined using the aluminium chloride method described by Abdel-Hameed (2009). The absorption at 440 nm was recorded after 2.5 h at 20^{o}C. Standard curve was established by using quercetin in methanol solution (0.0019-0.0312 mg/ml) under the same conditions. The concentration of flavonol expressed as mg quercetin equivalent/g of dried plant material (mg QE g^{-1}).was calculated using the following equation:

$$A = 34.046 \times FLL \text{ (mg QE } g^{-1}), R^2=0.9853$$

where A is the absorbance of the extracts in presence of an indicator and FLL is the flavonol content.

2.3.6. Determination of anthocyanin

Total anthocyanin content of the crude extracts were determined by a pH differential method in a 96 well microplate using a spectrophotometer (Versamax microtitre reader) as described by Lee *et al.* (2005) and Lee *et al*. (2008). Results expressed as mg cyanidin-3-glucoside equivalent per g dry plant material were obtained using equations 1 and 2 (Lee *et al*., 2005).

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

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

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

2.4. Antioxidant assays

2.4.1. DPPH free radical-scavenging method

Radical scavenging using the stable $DPPH^+$ as described by Brand-Williams *et al.* (1995) was used in evaluating the antioxidant activities of the extracts The method was modified to 96 well-microtitre plate format using extract solution (19–2000 µg/ml). The reductions in absorbance at 516 nm were monitored at 1, 5, 10, 15, 20, 25 and 30 min (A_t) using a spectrophotometer (Versamax). The negative control (A_o) was the absorbance of the DPPH radicals without antioxidant and the blank (A_b) was absorbance measured of respective samples treated the same way without DPPH radical.

2.4.2. ABTS free radical-scavenging method

Free radical scavenging activities of the extracts were evaluated using the ABTS cation decolourization method described by Re *et al*. (1999) with some modifications. The reduction in absorbance was measured after exactly 6 min of reaction (A_{t2}) and blank absorbance (A_{b2}) was prepared using the respective extracts without ABTS radical.

2.4.3. Ferric reducing antioxidant power (FRAP)

Transitional element reducing capacity of the extracts was evaluated by direct reduction of Fe^{3+} to Fe^{2+} as described by Berker *et al*. (2007) with some modification and expressed as ferric reducing antioxidant power (FRAP). The reaction medium (200 µl) made up of 50 µl of the test samples (19 to 2000 µg/ml) or positive controls (trolox and ascorbic acid, 9.5 to 1000 µg/ml), 100 µl of 1.0 M HCl, 20 µl of 1% of SDS, 30 µl of 1% of potassium ferric cyanide was incubated for 20 min at 50^{O}. The mixture was cooled to room temperature and finally 20 µl of 0.1% ferric chloride added. The absorbance was measured at 750 nm using a Versamax microtitre reader. The blank absorbance was prepared the same way except for the addition of ferric chloride. The reducing capacities were calculated as the slope from the line of best fit of the absorbance against concentration using the linear regression curve.

2.4.4. Linoleic acid lipid peroxidation inhibitory assay

The inhibition of the extracts on lipid peroxidation was determined according to the thiobarbituric acid method (Kishida *et al*., 1993). The Fenton reagent (Fe^{2+}+ $H_2O_2 \rightarrow Fe^{3+}$+$OH^{\bullet}$) was used to induce the linoleic acid emulsion peroxidation. In this assay, the aliquot contained 2.5 ml of linoleic acid solution in methanol, 150 µl of Tris HCl, 50 µl of ascorbic acid, 500 µl of extract (8-250 µg/ml) and 50 µl of $FeSO_4$. The mixture was incubated at 37^{o}C for 60 min in the dark and the reaction terminated by adding 516 µl of 40% trichloroacetic acid dissolved in 0.01% NaOH solution. The malondialdehyde (MDA) generated was measured by adding 1.6 ml of barbituric acid solution and the reaction mixture was heated at 95^{o}C for 15 min. The absorbance was recorded at 510 nm using the Versamax microtitre reader.

2.5. Antimicrobial activity assay

2.5.1 Minimum inhibitory concentration evaluation

The minimum inhibitory concentration (MIC) of the crude acidified 70% acetone extracts and different fractions against four bacterial strains and three fungal strains were determined using a broth microdilution technique (Eloff, 1998) for the bacterial assay and modified for fungal assay by Masoko *et al*. (2005).

2.6. Cyclooxygenase inhibitory assay

The anti-inflammatory capacities of the extracts were performed using both COX-1 (Jäger *et al*., 1996) and COX-2 (Noreen *et al*., 1998) by using radioactively labelled arachidonic acid.

2.7. Soybean 15-lipoxygenase inhibition

Inhibition of 15-lipoxygenase (15-LOX) was determined as outlined by Lyckander and Malterud (1992), using soybean lipoxygenase type 1-B (Sigma, South Africa). Measurements of increase in absorbance at 234 nm for 5 min after enzyme addition were done in 0.2 M borate buffer (pH 9.00) with linoleic acid (134 µM) as substrate and an enzyme concentration of 200 U/ml, using test substances dissolved in DMSO, and DMSO as blank. The conversion of linoleic acid to 13-hydroperoxylinoleic acid was recorded at 234 nm (room temperature) and compared to the appropriate standard solution, which did not contain the extracts. Every sample was tested in triplicate. The % Inhibition for different concentrations of the extracts was calculated.

2.8. Cytotoxicity assays

The toxic effects of the crude extracts on African green monkey kidney (Vero) cell viability was measured using the MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5 diphenyltetrazolium bromide] assay described by Mosmann (1983) with slight modifications (McGaw *et al*., 2007). Results were expressed as a percentage viability of the control cells and IC_{50} values were subsequently calculated.

2.9. Statistical analysis

Triplicate analyses were conducted and the mean ± SD values were obtained. The percentage radical scavenging capacities of the samples were calculated according to the following equation

$$\% \text{ Inhibition} = \{[A_o - (A_t - A_b)]/A_o\} \times 100$$

where A_o is absorbance of negative control, A_t is absorbance of samples at Time (t), A_b is absorbance of blank.

Log concentrations of the samples in the reaction medium were plotted against the percentage inhibition and EC_{50} indices were obtained by interpolation from sigmoidal dose-response

best fit curve using the GraphPad Prism version 5.04 for windows (GraphPad Software, San Diego California USA, http://www.graphpad.com).

3.0 Results and Discussion

3.1. Yield

The yields of the crude extracts were between 35-40% for the four species (Fig 1). This extraction method using combination of two immiscible extractants (acidified 70% acetone and hexane) extracted much more from the same species than acetone as reported by Eloff, (1999) (Cob 3.8%, Cop 1.4% and Cow 12.8%). The difference is explained by the large quantity of polar compounds extracted with this solvent mixture. The effectiveness of various extractants and solvent systems for fractionation and extraction conditions on the yield depends on the polarity of the constituents (Fig. 1). Both polar and non-polar solvents were used for the fractionation of the crude extracts. Removal of chlorophyll and non-polar constituents by hexane in the simultaneous extraction and fractionation process of leaf powder using mixture of hexane and acidified 70% acetone as extractant improved the extraction efficiency of phenolics. This process optimized the extraction condition and fractionation step.

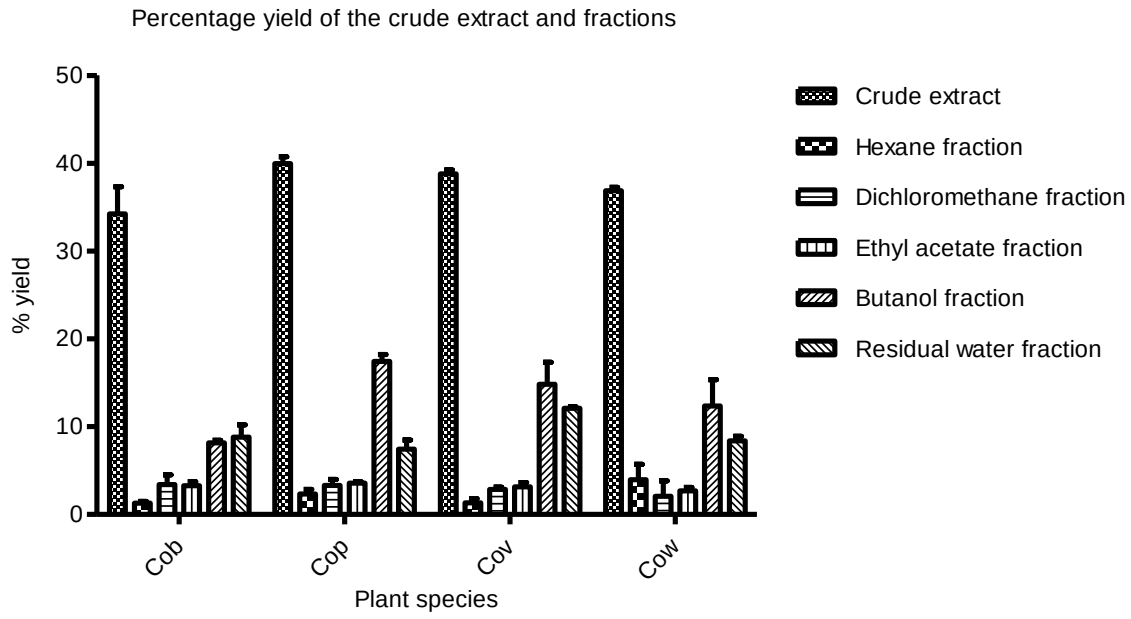


Fig. 1. Percentage yield of the crude and fractions of varied polarities of the *Combretum* species extracts. Cob = *Combretum bracteosum*, Cop = *Combretum padoides*, Cov = *Combretum vendae*, Cow = *Combretum woodii*.

Table 2
The minimum inhibitory concentration (µg/ml) for *Combretum* species extracts and fractions against four standard strains of bacterial and three clinical pathogenic fungal isolates

	Combretum bracteosum							*Combretum padoides*						
	E. c	*P. a*	*E. f*	*S. a*	*C. a*	*C. n*	*A. f*	*E. c*	*P. a*	*E. f*	*S. a*	*C. a*	*C. n*	*A. f*
Crude	312	312	156	625	625	78	312	156	156	78	156	312	39	156
Hex	39	78	312	78	625	625	312	19	78	19	19	312	156	156
DCM	156	312	312	312	312	625	156	19	39	19	39	19	625	78
ET $_{ac}$	312	625	156	156	156	39	156	312	625	156	156	312	19	78
ET $_{met}$	312	625	39	312	-	-	-	312	312	156	156	625	312	312
BU $_{ac}$	156	312	78	156	-	-	-	156	312	78	156	-	-	-
BU $_{met}$	625	156	156	78	156	19	312	39	19	78	156	156	312	156
W $_{met}$	>2500	312	312	312	1250	39	625	>2500	312	312	312	625	312	625
Genta	0.39	1.56	0.19	0.78	-	-	-	0.39	1.56	0.19	0.78	-	-	-
Amp B					6.25	3.12	0.7					6.25	3.12	0.78
	Combretum vendae							*Combretum woodii*						
	E. c	*P. a*	*E. f*	*S. a*	*C. a*	*C. n*	*A. f*	*E. c*	*P. a*	*E. f*	*S. a*	*C. a*	*C. n*	*A. f*
Crude	312	156	156	156	1250	78	156	625	625	156	312	1250	78	156
Hex	39	156	39	39	625	19	156	39	312	39	39	625	312	156
DCM	39	156	78	78	312	156	156	39	156	78	78	19	312	78
ET $_{ac}$	312	625	156	156	625	312	156	312	625	156	156	156	39	78
ET $_{met}$	156	156	78	78	78	78	156	312	312	78	156	312	312	78
BU $_{ac}$	156	312	78	156	-	-	-	156	312	78	156	78	156	78
BU $_{met}$	156	39	78	39	312	156	156	625	625	156	156	625	625	78
W $_{met}$	312	312	39	39	312	312	625	>2500	312	312	312	625	312	625
Gent	0.39	1.56	0.19	0.78	-	-	-	0.39	1.56	0.19	0.78	-	-	-
Amp B	-	-	-	-	6.25	3.12	0.7	-	-	-	-	6.25	3.12	0.78

N.B*: E. c* (*Escherichia coli*); *E. f* (*Enterococcus faecalis*); *S. a* (*Staphylococcus aureus*); *P. a* (*Pseudomonas aeruginosa*); *C. a* (*Candida albicans*); *C. n* (*Cryptococcus neoformans*); *A. f* (*Aspergillus fumigatus*); DCM (dichloromethane); ET $_{ac}$ (Ethyl acetate fraction soluble in acetone); ET $_{met}$ (Ethyl acetate fraction soluble in methanol); BU $_{ac}$ (Butanol fraction soluble in acetone); BU $_{met}$ (Butanol fraction soluble in methanol); W $_{met}$ (water fraction soluble in methanol), Gent (gentamicin) and Amp B (amphotericin B).

3.2. Antimicrobial assay

In general the polar fractions had higher antioxidant activity and the non-polar fractions had higher antimicrobial activities (Table 2). This confirms the results found on other *Combretum* species. The activity of the crude extract generally had the similar order of antibacterial activity compared to

acetone extracts of the same species determined earlier (Eloff, 1999). The most susceptible bacterial species to the crude extracts was *E. faecalis* with an MIC range of 78 to 156 µg/ml, followed by *E. coli* with an MIC range of 156 to 312 µg/ml. *S. aureus* growth was also inhibited by the extract with an MIC range of 156 to 625 µg/ml, and *P. aeruginosa* was the least susceptible organism within 24 h of exposure to the test samples. The non-polar fractions (hexane and DCM) had good antimicrobial activities worth further investigation. The MICs ranged between 19 and 312 µg/ml and the susceptibility of the bacteria to these fractions were in descending order of *E. coli* > *E .faecalis* > *S. aureus* > *P. aeruginosa*. The significant activity by the hexane and DCM fractions against the standard non-pathogenic diarrhoeal agents such as *E. coli, S. aureus,* and *E. faecalis* are noteworthy. It is encouraging that there appeared to be selectivity of certain fractions against different bacteria indicating that the activity may not be due to a general metabolic toxin.

C. neoformans was the most susceptible fungus with MIC ranging from 39 to 78 µg/ml. *C. padoides* extracts had the strongest activity with MIC of 39 µg/ml. *C. vendae* extracts had weak activity against *C. albicans* (MIC of 1250 µg/ml). *C. padoides* had some anticandidal activity on *C. albicans* (MIC of 312 µg/ml), followed by *C. woodii* (MIC of 625 µg/ml). The order of the broad spectrum of activity against the microorganism in the descending order was *C. padoides* > *C. vendae* > *C. woodii* > *C. bracteosum*. Antifungal activities of the fractions were also higher than the activity of the crude extracts. This makes sense because the extraction system also extracted a large quantity of inactive polar compounds. These results confirmed the antifungal activities of some *Combretum* species reported by Masoko *et al*., (2007). Acetone leaf extracts of 24 *Combretum* species also contained many different antifungal compounds based on bioautography (Masoko and Eloff, 2006).

Based on the phytochemical evaluation, some fractions contained a high concentration of polyphenolic compounds. Martini *et al*., (2004a) isolated seven antibacterial flavonoids from acetone leaf extracts of *C. erythrophyllum*. Several of these compounds had excellent antibacterial activities also against *Vibrio cholera* (Martini *et al*., 2004b). Some mechanisms of antimicrobial activity of phenolic compounds includes their ability to denature microbial proteins as surface-active agents (Sousa *et al*., 2006). Phenolic compounds may also react with cellular membrane components and impair the function and integrity of cells (Raccach, 1984). The reducing property of phenolics can influence the redox potential (E_h) of microbial growth, leading to growth inhibition (Jay, 1996).

Many people in rural areas of South Africa depend on medicinal plants to cure infectious diseases and GIT disorders including diarrhoea. Pending *in vivo* studies on efficacy, bioavailability and safety, extracts from some of these species may serve as therapeutic agents in infectious and other diseases with oxidative stress aetiology. These depend on use of appropriate extractants (organic reagents like ethanol) and administration of right dosage. Crude extracts and fractions from these species may also have activity against other diverse clinically important pathogens.

3.3. Antioxidant and free radical scavenging assay

The concentrations of crude extracts and fractions of various polarities required to reduce activities of DPPH and ABTS radicals by 50% (EC_{50}) were determined and presented in Tables 3 and

Table 3
DPPH radical scavenging activities of the *Combretum* species expressed as EC_{50} (µg/ml)

Test	*Combretum bracteosum*		*Combretum padoides*		*Combretum vendae*		*Combretum woodii*	
Samples	EC_{50}	R^2	EC_{50}	R^2	EC_{50}	R^2	EC_{50}	R^2
Crude	5.72±1.21	0.98	4.44±0.35	0.97	1.65±0.20	0.78	3.88±1.78	0.94
Hexane	85.04±10.56	0.96	12.65±1.3	0.94	16.88±2.66	0.95	9.41±3.51	0.92
DCM	20.53±0.40	0.98	3.33±0.30	0.94	4.10±0.31	0.94	2.16±0.28	0.89
ET_{ac}	7.76±0.68	0.94	0.44±0.06	0.90	1.02±0.14	0.97	1.24±0.13	0.96
ET_{met}	-	-	0.50±0.04	0.92	1.87±0.35	0.82	2.13±0.07	0.88
BU_{ac}	4.97±0.19	0.98	0.21±0.03	0.96	0.25±0.06	0.96	0.33±0.01	0.95
BU_{met}	-	-	0.67±0.11	0.97	0.84±0.12	0.94	1.00±0.03	0.96
W_{met}	35.65±4.78	0.86	0.84±0.15	0.93	0.96±0.12	0.95	1.10±0.07	0.96
Trolox	1.02±0.05	0.91	1.02±0.05	0.91	1.19±0.06	0.88	1.37±0.07	0.86
Asc acid	1.28±0.05	0.91	1.28±0.05	0.91	1.51±0.06	0.89	1.75±0.07	0.88

DCM (dichloromethane); ET_{ac} (Ethyl acetate fraction soluble in acetone); ET_{met} (Ethyl acetate fraction soluble in methanol); B_{ac} (Butanol fraction soluble in acetone); B_{met} (Butanol fraction soluble in methanol); Wmet = water fraction soluble in methanol

4 respectively. The lower the EC_{50}, the more effective the fractions are as free radical scavengers. Significant DPPH radical scavenging activity was observed with the EC_{50} value of the well-known controls (trolox 1.02±0.05–1.37±0.07 µg/ml and ascorbic acid 1.28±0.05–1.51±0.06 µg/ml). Highers antioxidant activities were found with the ethyl acetate (0.44±0.06 µg/ml for *C. padoides* acetone soluble to 2.13±0.07 µg/ml for *C. woodii* methanol soluble) and butanol sub-fractions (0.21±0.03 µg/ml

Table 4
ABTS radical scavenging activities of *Combretum* species expressed as EC_{50} (µg/ml)

Test samples	*Combretum bracteosum*		*Combretum padoides*		*Combretum vendae*		*Combretum woodii*	
	EC_{50}	R^2	EC_{50}	R^2	EC_{50}	R^2	EC_{50}	R^2
Crude	11.34±1.5	0.98	4.17±0.02	0.64	6.01±0.07	0.80	9.78±0.08	0.90
Hexane	1709±91.44	0.87	22.72±1.46	0.95	23.93±0.95	0.94	6.78±0.29	0.95
DCM	59.12±2.33	0.98	4.03±0.20	0.96	2.82±0.44	0.96	1.71±0.03	0.96
ET_{ac}	7.38±0.71	0.96	0.71±0.01	0.97	1.20±0.03	0.96	1.30±0.13	0.96
ET_{met}	-	-	1.31±0.06	0.95	6.72±0.30	0.977	4.12±0.36	0.96
BU_{ac}	22.57±1.03	0.98	0.43±0.03	0.92	0.77±0.03	0.97	0.83±0.06	0.96
BU_{met}	-	-	0.55±0.04	0.96	0.60±0.03	0.93	0.61±0.03	0.94
W_{met}	190.64±16.3	0.98	1.47±0.82	0.94	4.26±0.12	0.95	5.73±1.50	0.96
Trolox	1.30±0.21	0.97	1.30±0.21	0.97	1.32±0.21	0.96	1.27±0.21	0.96
Asc acid	1.31±0.09	0.95	1.31±0.09	0.95	1.32±0.09	0.95	1.28±0.08	0.95

DCM (dichloromethane); ET_{ac} (Ethyl acetate fraction soluble in acetone); ET_{met} (Ethyl acetate fraction soluble in methanol); BU_{ac} (Butanol fraction soluble in acetone); BU_{met} (Butanol fraction soluble in methanol); W_{met} = water fraction soluble in methanol

for *C. padoides* acetone soluble to 1.00±0.03 µg/ml for *C. woodii* methanol soluble) for all the tested *Combretum* species except C. *bracteosum*. These fractions are more potent antioxidative agents than the well known controls. The crude extracts also had good DPPH radical scavenging properties with *C. vendae* being the most effective with EC_{50} of 1.65±0.20 µg/ml (R^2 = 0.7855), followed closely by *C. woodii* with EC_{50} of 3.88±1.78 µg/ml (R^2 = 0.9405) at steady state. The EC_{50} for *C. padoides* was 4.44±0.35 µg/ml (R^2 = 0.9704) and *C. bracteosum* had the lowest antioxidant activity with EC_{50} of 5.72±1.78 µg/ml (R^2 = 0.9806). The DPPH radical scavenging kinetics of the crude extracts and fractions is presented in Fig. 3.

The $ABTS^+$ radical scavenging activity of the extracts and fractions followed the same trends as the DPPH assay. The butanol sub-fractions were more effective as radical scavengers than the controls (trolox and ascorbic acid). The mechanism of anti-oxidation involves electron transfer from ABTS to the test sample, indicating that some of the extract components are good electron acceptors. Although the two assays may not have biological significance, they served as rapid and efficient measures of phytochemical antioxidative capacity *in vitro*. The evaluation methods can be used to

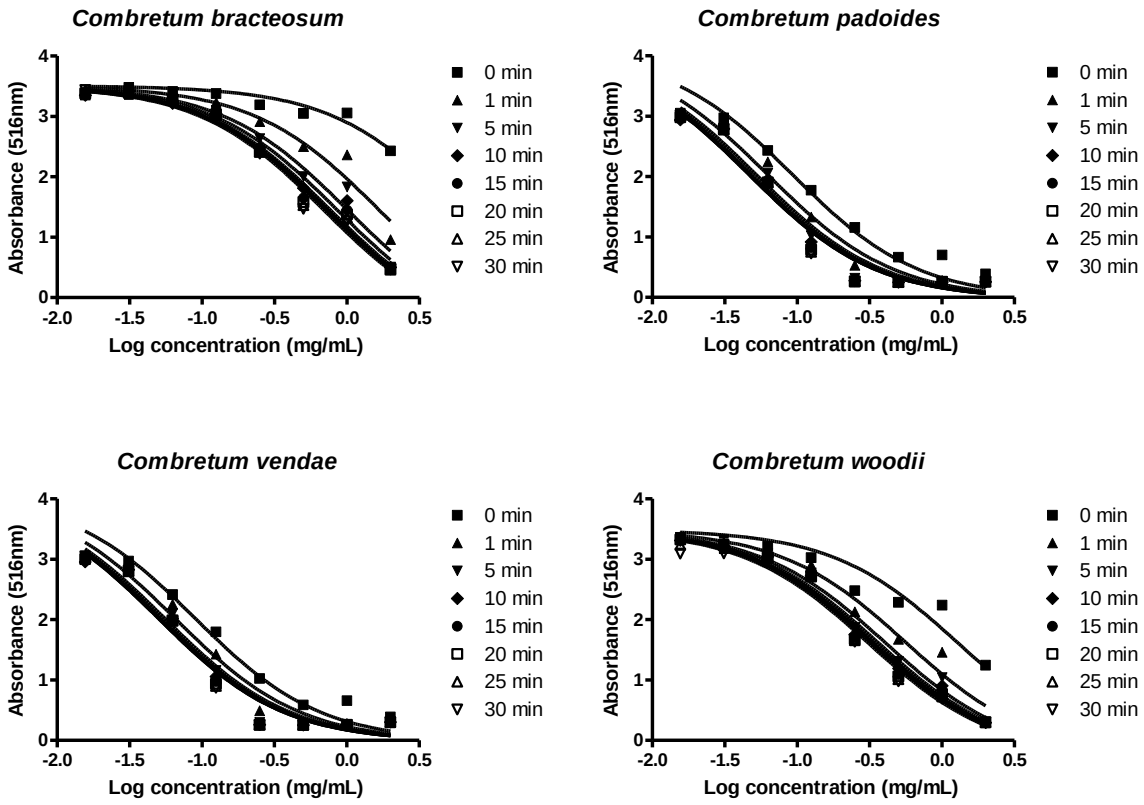


Fig. 3. Sigmoidal dose-dependent response of the crude extracts for DPPH radical capacity as absorbance against Log concentration

categorize antioxidative potential of medicinal plants and also for bioactivity-guided isolation of compounds with high antioxidant activities.

Oxidants are important parts of the body system serving as endogenous microbicidal agents in the phagocytosis process and as cellular messengers in numerous metabolic pathways. The activation of phagocytic cells such as macrophages, neutrophils and eosinophils leads to the generation of superoxide anions (MacNee, 2001). Superoxide is rapidly reduced by the superoxide dismutase enzyme to generate membrane permeable hydrogen peroxide. The release of H_2O_2 may lead to the formation of more stable oxidants such as hypochlorous acid, chloramines, and toxic aldehyde derivatives from lipid peroxidation of mucosal epithelial membrane. In diarrhoeal aetiology, oxidative molecules act directly as secretagogues (Gaginella, 1995), metabolites of intestinal lipid peroxidation acting as toxins (Xu *et al*., 2007) and/or, inflammation and inflammatory metabolites resulting in oxidative damage of the intestine (Gelberg, 2007). The body mechanisms used in

removing deleterious effects of oxidation include small antioxidant molecules like ascorbate, polyunsaturated fatty acids or sugars (mainly mannitol). Some endogenous ROS-scavenging enzymes, such as superoxide dismutases (SOD), catalase, and various peroxidases are also detoxifying agents (Heike-Steiling *et al*., 1999).

Table 5
Ferric reducing antioxidant capacities of *Combretum* species expressed as slope of linear regression curve

Test samples	*Combretum bracteosum*		*Combretum padoides*		*Combretum vendae*		*Combretum woodii*	
	Slope	R^2	Slope	R^2	Slope	R^2	Slope	R^2
Crude	9.4±0.32	0.97	6.36 ± 0.26	0.96	3.310 ± 0.25	0.89	4.983 ± 0.21	0.96
Hexane	0. 27±0.04	0.71	16.96 ± 0.91	0.94	14.28 ± 0.72	0.95	24.02 ± 1.79	0.89
DCM	1.6±0.10	0.91	22.68 ± 1.04	0.95	27.84 ± 1.66	0.93	25.91 ± 2.98	0.77
ET_{ac}	-	-	54.15 ± 4.87	0.85	49.87 ± 2.91	0.93	45.89 ± 3.87	0.86
ET_{met}	2.7±0.40	0.67	47.80 ± 4.68	0.82	30.44 ± 1.20	0.97	31.08 ± 1.43	0.95
BU_{ac}	-	-	44.11 ± 4.06	0.84	42.59 ± 3.81	0.85	39.87 ± 3.22	0.87
BU_{met}	5.2±0.25	0.95	40.54 ± 2.62	0.92	41.53 ± 1.98	0.95	41.19 ± 1.28	0.98
W_{met}	11.0±0.37	0.98	9.596 ± 0.58	0.92	3.597 ± 0.37	0.81	3.436 ± 0.67	0.54
Trolox	54.74 ± 2.13	0.97	54.74 ± 2.13	0.97	54.74 ± 2.13	0.97	54.74 ± 2.13	0.97
Asc acid	56.48 ± 1.50	0.99	56.48 ± 1.50	0.98	56.48 ± 1.50	0.98	56.48 ± 1.50	0.98

DCM (dichloromethane); ET_{ac} (Ethyl acetate fraction soluble in acetone); ET_{met} (Ethyl acetate fraction soluble in methanol); BU_{ac} (Butanol fraction soluble in acetone); BU_{met} (Butanol fraction soluble in methanol); W_{met} = water fraction soluble in methanol

The reducing power of the extracts and fractions presented in Table 5 indicated good activity against Fe^{2+} which is capable of initiating the Haber-Weiss reaction to generate $OH^{\cdot}$ from H_2O_2 in a self-perpetuated chain reaction. The results were presented as the slope of the best fit linear regression curve and the steeper the slope the more active the extracts or fractions are as reducing agents.

Among the transition metals, iron is known as the most important lipid pro-oxidant due to its high reactivity. The ferrous state of iron accelerates lipid oxidation by breaking down hydrogen and lipid peroxides to reactive free radicals via the Fenton reaction. The presence of excessive free ferrous ion in the intestine results in abdominal discomfort due to nausea, vomiting, altered bowel motility and black stools (Rimon *et al*., 2005). This is due to the reaction of Fe^{2+} with H_2O_2 and O_2^{-} release from activated neutrophils via the Haber-Weiss process, generating OH^{-} and Fe^{3+}. A

weakened endogenous antioxidant system may increase the susceptibility of the intestinal mucosa to peroxidative damage and microbial infection resulting in diarrhoea (Chen *et al*., 2007). A high level of Fe^{2+} damages intestinal barrier integrity such as tight junctions which control movement of material across the epithelial cells. The intestinal barrier dysfunction consequently results in reduced absorptive capacity *in vivo* (Chen *et al*., 2007) leading to osmotic diarrhoea. The agents that can attenuate the action of these bivalent metal ions have been classified as secondary antioxidants which retard the rate of radical initiation reaction by the elimination of initiators (Vaya and Aviram, 2001). The results of this study indicate that the extract and polar fractions can modulate hydroxyl radical generation by their strong ferric reducing ability.

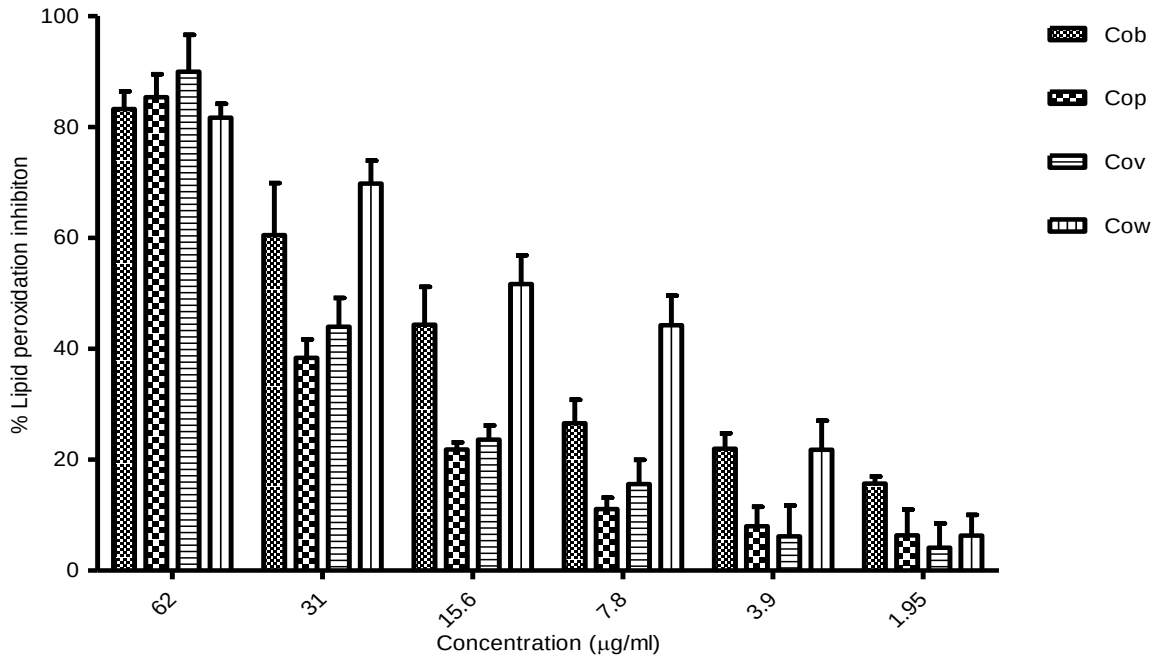


Fig. 2. Concentration-dependent lipid peroxidation inhibition of linoleic acid emulsion
Cob = *Combretum bracteosum*, Cop = *Combretum padoides*, Cov = *Combretum vendae*, Cow = *Combretum woodii*

The dose-dependent lipid peroxidation (LPO) inhibition of linoleic acid emulsion by the crude extracts is presented in Fig. 2. All the extracts had good inhibitory activity against lipid peroxidation as more than 80% of the peroxidation was inhibited (the maximum concentration of the extract was 64 µg/ml). The results indicate that the extract constituents contain hydroxyl groups capable of accepting electrons and removing $OH^{\cdot}$ formed in the Fenton reaction ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\cdot}$) process. The effect of lipid peroxidation and its products such as malondialdehyde (MDA), 4-hydroxy-2-nonenal (HNE) and 4-hydroxy-2-hexenal (HHE) on intestinal epithelial mucosa membrane are the major cause

of cellular dysfunction in the GIT. The effects of LPO include changes in membrane fluidity and permeability, altered ion transport across the intestinal epithelium, and inhibition of metabolic processes (Nigam and Schewe, 2000). These effects are some of the mechanisms or pre-disposing conditions that initiate and propagate diarrhoeal diseases. The high activity of extracts of these four species against lipid peroxidation may be related to their traditional use as antidiarrhoeal agents. Through these processes, they are capable of modulating the direct effects of radical, lipid peroxidation products as well as the resultant inflammation and diarrhoea.

Exogenous antioxidants from dietary intake, supplements or supportive drugs may be useful to promote healing. Therefore, the crude extracts and the potentiated antioxidant fractions of the *Combretum* species tested may be useful in attenuating the injurious role of oxidants in diarrhoeal diseases.

3.4. Anti-inflammatory assays

The phenolic-enriched crude extracts of *C. padoides*, *C. vendae* and *C. woodii* had no cyclooxygenase inhibitory activity against COX-1 and 2 enzymes. However, the anti-inflammatory activities of these extracts cannot be ruled out as they may involve other inflammatory mediators. In diarrhoeal diseases, ROS and RNS are known to activate many pro-inflammatory cytokines (interleukins and TNF-α), 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).

The extracts had variable lipoxygenase inhibitory capacity with LC_{50} ranges from 14.36±4.87 to 30.80±5.22 µg/ml. The highest activity was obtained from *C. woodii* with an LC_{50} value of 14.36±4.87 µg/ml followed by *C. vendae* with an LC_{50} of 22.34±1.70 µg/ml while the least active was *C. padoides* with an LC_{50} value of 30.80±5.22 µg/ml. The soybean 15-LOX inhibitory activity may be an indication of anti-inflammatory potential through this mechanism. Percentage inhibition of soybean 15-LOX enzyme by the crude extracts of the *Combretum* species is presented in Fig. 4.

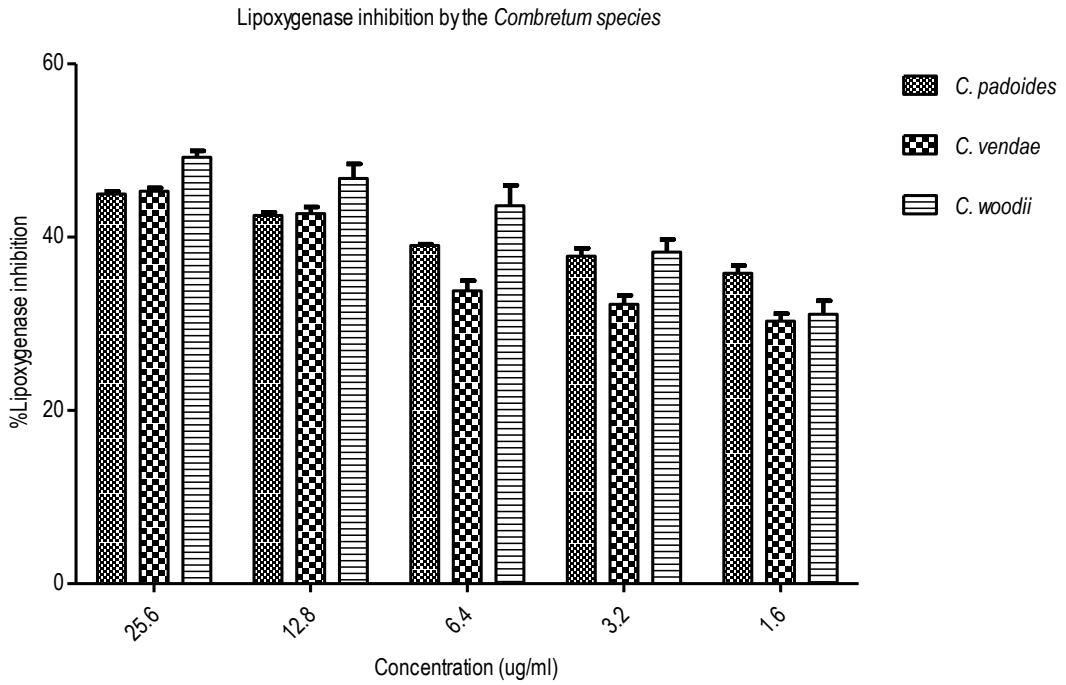


Fig. 4. Percentage soybean15-lipoxygenase inhibition by *Combretum species*

Although the phenolic-enriched extracts of *C. padoides, C. vendae* and *C. woodii* may not have directly affect the COX enzyme activity, the appreciable free radical, ferric reducing properties and inhibition of lipid peroxidation may influence the inflammation process.

3.5. Phenolic constituents of the crude extracts

The results of the quantitative evaluation of the phenolic constituents of the crude extracts determined by colorimetric methods are presented in Table 6. The total polyphenolic content of the extracts ranged from 467±15.8 mg GAE/g (*C. padoides)* to 184±5.2 mg GAE/g (*C. bracteosum).* There was a good correlation (R^2 = 0.9712) with the total tannin content which varied between 359.4 ± 6.2 mg GAE/g (*C. padoides*) to 84.7± 6.5mg GAE/g (*C. bracteosum)*. The Folin-Ciocalteau assay used to determine the total polyphenolic content was based on the redox reaction; however, the reaction is prone to interference by non-phenolic components such as ascorbic acid (Dykes and Rooney, 2006). In addition, hydrolysable gallotannin which ranged between 305 mg GAE/g (*C. padoides*) to 76 mg GAE/g (*C. bracteosum*) correlated well with the total polyphenol (R^2 = 0.8509) and total tannin (R^2 = 0.9388) content. However, *Combretum bracteosum* contains a higher quantity of condensed tannin compared to the quantity of hydrolysable tannin. There was no correlation between the condensed tannin (R^2 = 0.0622) and total polyphenolics, and also there was no

Table 6
Polyphenolic content of the crude extracts extracted with acidified 70% acetone in aqueous solution

Properties	*Combretum bracteosum*	*Combretum padoides*	*Combretum vendae*	*Combretum woodii*
Total yield (mg/g)	285.4±15.3	513.2±35.7	546.2±29.5	392.5±30.3
Total polyphenols (mg GAE/g)	184.1±5.2	467±15.8	444.2±15.4	240.8±30.3
Non-tannin polyphenol (mg GAE/g)	99.3±19.4	107.6±8.6	155.8±6.4	94.5±6.8
Total tannin (mg GAE/g)	84.7±6.5	359.4±6.2	288.4±8.*3*	145.5±4.2
Condensed tannin V/H (mg CE/g)	121.9±5.5	82.7±7.7	77.1±7.67	48.3±1.32
Proanthocyanidin B/H (mg LE/g)	87.98±5.7	24.1±2.4	24.4±3.6	11.7±2.1
Gallotannin (mg GAE/g)	76.8±1.06	305.8±19.09	197.6±12.78	83.5±2.51
Anthocyanin (mg Cyn-3-glu)	7.3±0.34	6.03±0.53	1.58±0.001	3.96±0.09
Total flavonoids (mg QE/g)	17.05±0.84	40.95±2.34	15.35±0.97	14.7±0.53
Flavonol (mg QE/g)	0.984±0.004	0.086±0.001	0.094±0.007	0.274±0.06

N. B: mg = milligram, g = gram, GAE = gallic acid equivalent, CE = catechin equivalent, LE = leucoanthocyanidin equivalent, Cyn-3-glu = cyaniding-3- glucose, QE = quercetin

correlation between the condensed tannin (R^2 = 0.0737) and the total tannin. Condensed tannins were estimated by the vanillin/HCl method involving the condensation of the aromatic aldehyde vanillin (4-hydroxy-3-methoxybenzaldehyde) with monomeric flavanol and their oligomers to form adducts that absorb at 500 nm. However, other non-tannin phenolics with the same functional group may interfere to give variable values. A lack of an appropriate standard for the condensed tannin determination was also a major setback due to heterogeneity of condensed tannin and the use of vanillin as indicator may result in unrealistic values (Schofield *et al*, 2001).

The proanthocyanidin content was determined by the butanol/HCl method and had no correlation with total polyphenolics, and total tannin respectively. However, there is no appropriate standard to compare the result. The reaction is sensitive to water content, transition metal ions and the ratio of the butanol/HCl to sample in the reaction medium, therefore unreliable data may be obtained if proper precautions are not taken (Schofield *et al,* 2001).

The total flavonoids ranged between 40.95 mg QE/g (*C. padoides*) to 14.7 mg QE/g (*C. woodii*) and had a low correlation with total polyphenol content (R^2 = 0.3467). However, the flavonol content was highest in *C. bracteosum* of 0.984 mg QE/g, followed by *C. woodii 0.*274 mg QE/g, *C. vendae* 0.094 mg QE/g and *C. padoides* 0.086 mg QE/g and have some correlation (R^2 = 0.6895) with the total polyphenolic content of the extracts. There was also a very correlation (R^2 = 0.2061)

between the anthocyanin, which ranged between 7.3 mg Cyn-3-glu (*C. bracteosum*) to 1.58 mg Cyn-3-glu (*C. vendae*) and the total polyphenols. The compositional data presented here are estimations of their chemical equivalent activities. Different compounds may contribute differently or have different kinetics to the reagent used in the evaluation. Also only the extractable components were determined since some may have been bound strongly to the plant matrix. Despite the problems commonly associated with the colorimetric methods of evaluating the phytochemical constituents of extracts, it still remains among the easiest and simplest quantification techniques in plant chemistry.

There was a good correlation (R^2 = 0.9907) between the total polyphenolic content and the radical scavenging activity in gallic acid equivalents. Similar relationships were obtained between the total tannin (R^2 = 0.9488) and gallotannin (R^2 = 0.8667) and the radical scavenging activity in gallic acid equivalents, respectively. However, there were no good correlations between the radical scavenging activity and condensed tannin (R^2 = 0.0259), proanthocyanidin (R^2 = 0.2375), anthocyanin content (R^2 = 0.1774), total flavonoid content (R^2 = 0.3503) and flavonol content (R^2 = 0.6004).

Other authors reported a low correlation between DPPH radical scavenging activity, and flavonoids and their derivatives (Vundac *et al*., 2007). The antiradical ability of flavonoids depends on structural pre-requisites, especially on the number and arrangement of hydroxyl groups, the extent of structural conjugation and the presence of electron-donating or electron-accepting substituents on the ring structure (Cieslik *et al*., 2006). The correlation between chemical constituents and the free radical scavenging activity may give an insight into the antioxidant mechanisms of the extracts, which could be due to hydrogen donation.

From the compositional data (Table 6), the main compounds present in the *Combretum* species are hydrolysable gallotannins except *C. bracteosum* which contains a significant quantity of condensed tannins. The basic structural unit of gallotannin is the polyol-D-glucose, esterified by gallic acid at its hydroxyl group to give the β-pentagalloyl-D-glucose. It could be deduced that the hydrolysable gallotannin may be responsible for the high activity of the extracts. Polyphenolics exert therapeutic activities against infectious and degenerative ailments via mechanisms such as antioxidant, enzyme modulation, immunomodulation and anti-inflammatory activity. Therefore, the characteristic high content of hydrolysable tannins in the *Combretum* species, though not exclusive of other compounds, may play a role in treating diarrhoea. This would be useful in treating acute

diarrhoea but would have limited use in combating diarrhoea in animal feedlots as aprophylactic because of the negative nutritional aspects related to tannins due to its interaction with proteins.

3.6. Cytotoxicity

To evaluate the cellular safety of the plant extracts as antidiarrhoeal agents, we determined the *in vitro* toxicity using the MTT cytotoxicity assay on Vero monkey kidney cells culture with berberine as positive control and DMSO as negative control. The percentage cell viability for each extract at different concentrations is presented in Fig 5. All the extracts except *C. bracteosum* had a

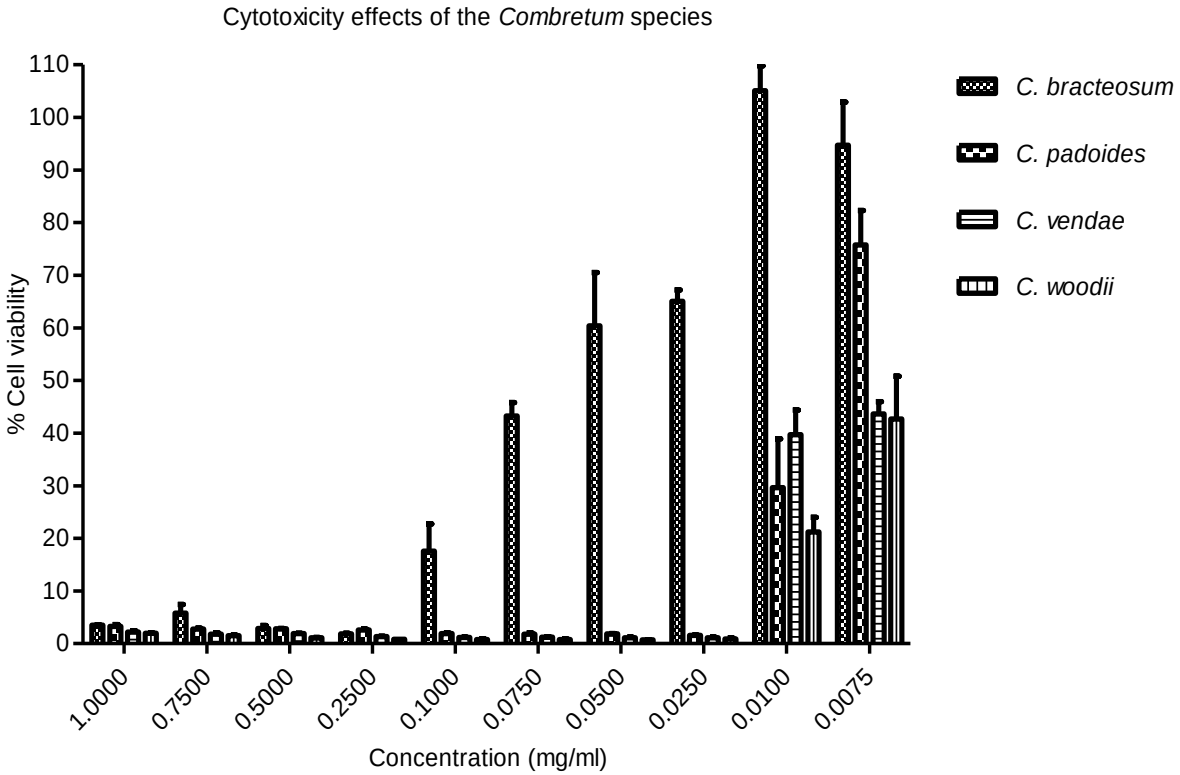


Fig. 5. Concentration-dependent effect of the *Combretum* species on the Vero cell viability

degree of toxicity at a relatively low concentration value (10 µg/ml). The 50% cytotoxicity concentration (LC_{50}) varied from 3.51±2.03 to 48.81±6.15 µg/ml. The least toxic extract was that of *C. bracteosum*, which had an LC_{50} of 48.81±6.16 µg/ml while the most toxic was the *C. woodii* extract with LC_{50} of 3.51±2.04 µg/ml. *Combretum padoides* and *C. vendae,* had LC_{50} values of 9.04±0.22 and 5.70±1.23 µg/ml respectively. Berberine chloride was toxic with an LC_{50} of 1.89±0.008 µg/ml. 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. *C. woodii* extracts are toxic to poultry in an *in*

vivo anticoccidial test at a concentration of 160 mg/kg (Naidoo *et al*, 2008). Furthermore, several cytotoxic and anti-tumour derivatives of stilbenoids (Fyhrquist, 2007) such as combretastatin A and combretastatin B5 (LC_{50} value of 10 µg/ml) have been isolated from the genus *Combretum*.

4. Conclusion

From the results presented in this study, some of the crude extracts and fractions of *Combretum* species studied have good pharmacological activities that may be relevant in treatment of infectious and gastrointestinal diseases. In the *in vitro* anti-inflammatory assay, crude extracts from these plants were not active against COX 1 and COX 2 enzymes but conversely some had inhibitory activity against LOX enzyme. From the compositional data, gallotannin and other polyphenolic compounds are the predominant constituents that correlate with antioxidant activity. This study highlights the biological potential of the polyphenolic constituents of these *Combretum* species. Use of crude extracts from these plants should take into account the *in vitro* toxicity. Therefore, for these plants to be useful as therapeutic agents, *in vivo* toxicity studies of the crude extracts and fractions are necessary. Also, *in vivo* antioxidative effect of some fractions against oxidative tissue damage and anti-infective properties merit further investigation.

Acknowledgements

The Medical Research Council and the National Research Foundation (NRF); South Africa provided funding. The plant leaves were collected from the Manie van der Schijff Botanical Garden (University of Pretoria). Lorraine Middleton and Magda Nel of the Botany Department assisted in identification of the trees and preparing of herbarium specimens.

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