Anti-inflammatory, anticholinesterase and antioxidant activity of leaf extracts of twelve plants used traditionally to alleviate pain and inflammation in South Africa

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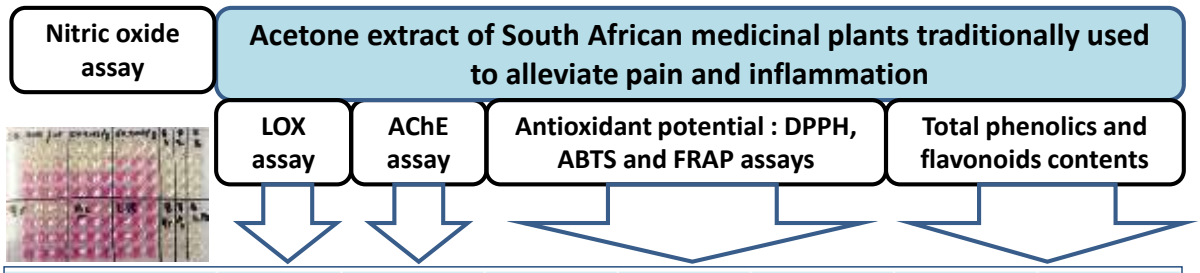
Graphical abstract



Plant name	15-LOX IC_{50} (µg/mL)	AChE IC_{50} (µg/mL)	DPPH IC_{50} (µg/mL)	ABTS IC_{50} (µg/mL)	FRAP IC_{50} (µg Fe (II)/g)	TPC (mg GAE/g)	TFC (mg QE/g)
E. rigida	62.99±1.43	487.44±0.17	171.75±3.46	234.83±3.18	94.07±3.26	103.28±4.26	6.34±2.77
J. curcas	41.50±1.24	382.89±2.01	137.08±4.03	115.23±2.66	68.17±4.44	130.12±17.06	60.87±0.54
U. nitida	60.50±3.13	475.12±9.12	125.86±3.85	28.81±2.45	177.32±12.55	137.30±46.40	15.37±0.37
Z. rivularis	-	362.52±12.89	74.05±4.80	36.52±1.57	286.31±67.93	182.79±27.97	46.88±10.41
E. undulata	-	450.34±18.71	31.66±0.58	32.67±0.45	274.19±29.36	129.78±32.58	35.16±2.49
A. laxiflora	46.03±2.10	364.12±2.39	17.19±1.02	18.53±1.42	438.42±15.55	147.95±30.18	13.84±1.47
M. mesozygia	58.62±0.15	-	15.85±0.81	271.86±6.43	127.34±18.34	181.49±13.12	13.75±2.44
E. magaliesmontanum	63.25±9.11	160.19±1.81	10.80±0.56	12.22±3.24	76.00±3.21	100.89±4.00[e]	68.43±2.04
L. leucocephala	43.99±0.79	118.23±4.40	9.86±0.59	9.05±0.81	289.25±82.87	258.40±7.45	159.61±12.79
L. javanica	44.99±2.37	363.74±11.16	7.98±0.84	18.18±1.50	579.49±32.21	427.53±88.63	80.72±4.93
B. africana	37.25±0.88	386.11±16.66	3.55±0.32	3.12±0.54	231.07±91.43	150.82±17.43	72.80±21.42
C. zeyheri	45.46±0.75	398.62±2.41	3.52±0.46	4.64±0.48	95.98±5.30	234.56±63.70	64.36±5.86
Trolox	nd	nd	3.14±0.10	6.05±0.24	nd	nd	nd
Ascorbic acid	nd	nd	1.95±0.04[l]	3.57±0.18	nd	nd	nd
Quercetin	35.85±0.18	nd	nd	nd	nd	nd	nd
Indomethacin	-	nd	nd	nd	nd	nd	nd
Eserine	nd	4.94±0.05	nd	nd	nd	nd	nd

Abstract

Ethnopharmacological relevance

Oxidative stress and inflammatory conditions are among the pathological features associated with the central nervous system in Alzheimer's disease. Traditionally, medicinal plants have been used to alleviate inflammation, pains and also other symptoms possibly associated with Alzheimer's disease. Therefore, the present study was designed to determine the *in vitro* anti-inflammatory, antioxidant and anticholinesterase activity of twelve South African medicinal plants traditionally used to alleviate pain and inflammation.

Materials and Methods

Nitric oxide (NO) production in LPS-activated RAW 264.7 macrophages and 15-lipoxygenase (LOX) inhibitory assay were used to evaluate the anti-inflammatory activity. Acetylcholinesterase inhibition was assessed by using a modification of the Ellman's method. Antioxidant activity, total phenolic and total flavonoids contents were determined using standard *in vitro* methods.

Results

The extract of *Burkea africana* had the highest anti-15-lipoxygenase activity with 85.92% inhibition at 100 µg/mL. All the extracts tested inhibited nitric oxide (NO) production in a dose dependant manner in LPS-stimulated RAW 264.7 macrophages. However, extracts from *Leucaena leucocephala, Lippia javanica* inhibited the production of NO by 97% at a concentration of 25 µg/mL. In addition, both *Leucaena leucocephala* and *Englerophytum magaliesmontanum* had strong activity against acetylcholinesterase with IC_{50} values of 118 µg/mL and 160 µg/mL respectively. Hight levels of phenolics and flavonoids were found in *Leucaena leucocephala, Lippia javanica* and *Burkea africana.* The correlation with antioxidant activities was not strong indicating that other metabolites may also be involved in antioxidant activity.

Conclusions

The results obtained in this study validate the use of leaf extracts of these plants in South African traditional medicine against inflammation. Extracts of these plants species might be of value in the management of various diseases emerging from oxidative stress and related degenerative disorders.

Key words: Medicinal plants, anti-lipoxygenase, nitric oxide production inhibition, anticholinesterase, antioxidant.

1. Introduction

In Africa, plants have always been and still remain a vital source of therapeutics for various illnesses such as inflammation, cognitive deficit and oxidative stress related disorders. Neurodegenerative disorders such as Alzheimer's and Parkinson's diseases are characterized by progressive memory loss and an impairment of cognitive function due to a reduced cholinergic activity in brain (Mattson, 2004). Besides the neuro-pathologic hallmarks of this disease, oxidative stress has also been recognized as a key factor in its pathogenesis (Lee et al, 2012). Excess free radicals generated in the body attack most cellular macromolecules such as proteins (enzymes), lipids and DNA leading to oxidative stress. Oxidative and inflammatory processes are among the pathological features associated with the central nervous system in Alzheimer's disease (AD) (Houghton et al, 2007). The excess of reactive oxygen species (ROS) generated, leads to inflammation by stimulating release of cytokines and activation of enzymes such as lipoxygenases (LOXs) from inflammatory cells. LOX is involved in provoking several inflammation related diseases (Dobrian et al, 2011). These enzymes also play an important role in inflammation, since they are involved in the biosynthesis of inflammatory lipid mediators, such as leukotrienes and prostagladins, and their inhibition is considered as one of the targets for the prevention of diseases, whose development is linked to oxidative stress and inflammation (Radmark and Samuelsson, 2007). Chronic inflammation in the brain is a pathological feature of Alzheimer's disease (AD) and in this process, large amounts of pro-inflammatory substances such as nitric oxide (NO) are produced (Wang et al, 2013). Therefore, the use of antioxidant

substances that scavenge and eradicate ROS may prevent or minimize these oxidation-related diseases. Selkoe (2005) suggested the use of antioxidants and free radical scavengers as possible treatment options for certain features of Alzheimer's disease. Moreover, targeting inhibitors of LOX and AChE may be an indication of potential therapeutic use in treatment of cognitive dysfunction. The efficacy of natural antioxidants and anti-inflammatory drugs in treating inflammatory and neurodegenerative disorders has been widely documented (Morris et al, 2002; Stuchbury and Munch, 2005; Fusco et al, 2007). Plant based antioxidant compounds play a defensive role by preventing the generation of free radicals and hence could be beneficial in alleviating the diseases caused by oxidative stress (Fusco et al, 2007). In this regard, plants have been a source of medicinal agents for thousands of years, and an impressive number of modern drugs have been isolated from natural sources, many based on their use in traditional medicine and there is an increasing interest in the therapeutic use of natural products especially those derived from plants (Rates, 2001). In the African Herbal Pharmacoepia (Brendler et al. 2010) numerous plants have been used to treat various ailments, including inflammation and neuropharmacological disorders Although there is an important local ethnobotanical bibliography describing the most frequently used plants in the treatment of conditions consistent with sepsis and other diseases, there are few experimental studies, which validate the therapeutic properties of these plants. According to Iwalewa et al (2007), more than 115 plant species of 60 families are used in South Africa for treating pain-related inflammatory disorders in humans and animals. An ethnopharmacological investigation and bioassay-guided isolation of active compounds may lead to the discovery of more effective and safer therapeutic agents or plant extracts to treat diseases linked to oxidative stress and inflammation. Using tree leaves either for the isolation of bioactive compounds or therapeutically useful extracts will leads to the

sustainable use of these genetic resources (Pauw and Eloff, 2014). Based on this rationale, we investigated the 15-lipoxygenase, NO production and AChE inhibitory activity as well as the free radical scavenging capacity of extracts of selected indigenous plants in South Africa that have been used in the treatment of inflammation and associated pain (Table 1).

2. Materials and Methods

2.1. Plant material and extraction

The leaves of plants were collected in the Pretoria National Botanical Garden. We focused on leaves because it is a sustainable resource and because even where bark has been traditionally used, leaf extracts had a higher activity (Eloff, 2001; Shai et al., 2009) The identity of the plant material was confirmed by the curator and voucher specimens were placed in the HGWJ Schweickerdt Herbarium of the University of Pretoria (Table 1). Collected leaves were dried at room temperature in a well-ventilated room and ground to a fine powder in a Macsalab Mill (Model 2000 LAB Eriez). One gram of each plant was extracted in 10 mL of acetone, (technical grade, Merck) in a polyester centrifuge tube. Acetone was selected as extractant because many studies in our group have shown that it yielded higher activities in a variety of indications (Eloff 1988, Eloff et al., 2005). It should also be kept in mind that aqueous extracts of traditional healers using non-sterile conditions could lead to microbial growth leading to solubilizing non-polar metabolites. The tube was vigorously shaken for 30 min on an orbital shaker, then centrifuged at 4000 x g for 10 min and the supernatant was filtered using Whatman No.1 filter paper before being transferred into pre-weighed glass containers. This was repeated thrice on the same plant material and the solvent was removed by evaporation under a stream of air in a fume hood at room temperature to produce the dried extract.

Table 1: Characteristics of the twelve South African plants traditionally used to alleviate pains and inflammations investigated.

Plant name (FAMILY)	Other name	Part used	Traditional use	Voucher number
Ehretia rigida (Thunb.) Druce (BORAGINACEAE)	Puzzle bush (Eng.); umHlele (Zulu); deurmekaarbos (Afr.); Morobe (Northern Sotho); iBotshane (Xhosa); Mutepe (Venda)	Roots	Small cuts in the skin, over the abdomen and chest to relieve pains, gall sickness in cattle, protect huts and crops from hail. (Carruthers, 1997).	PMDN 340
Combretum zeyheri Sond (COMBRETACEAE)	Large-fruited bush willow, large-fruited combretum, Zeyher's bush willow fluisterboom (Eng.), Nikbaase-klapper, raasblaar, raasbos, raasklapper, wurmhout (Afr.) umbondwe- mhlope, umbondwe wasembundwini (Zulu)	Bark, stems, roots	Circulatory and digestive System Disorders, Genitourinary System Inflammation, Pain, Poisonings, Diahrea, cancer (Fyhrquist et al, 2008)	PMDN 482
Euclea undulata Thunb. (EBENACEAE)	Common guarri guarri, thicket euclea (Eng.), gewone ghwarrie, ghwarriebos (Afr.); gwanze, inkunzane, umbophanyamazane, umshekisane, Umtshekizane (Zulu)	Leaves, roots, barks.	Diabetes. Diarrhea, stomach, tonsillitis, Enemata, purgation, headache, toothache, pains (De Winter, 1963).	PMDN 415
Alchornea laxiflora (Benth.) Pax & K. Hoffm. (EUPHORBIACEAE)	Lowveld bead-string (Eng.)	Leaves, stem, branchlets	Anti-tumour, inflammation, infectious diseases, teething problems, chewing sticks (Farombi et al, 2003; Sandjo et al, 2011).	PMDN 392
Jatropha curcas L. (EUPHORBIACEAE)	Physic nut, purging nut tree, pig nut; fig nut; barbados nut; pinhoen oil (the seed-oil). (Eng.)	All parts	Mouth infections, veterinary ailments, anticancer, skin diseases, piles and sores among the domestic livestock, malaria, rheumatic and muscular pains, snake venom (Thomas, 2008)	PMDN 606
Leucaena leucocephala (Lam) De Wit (FABACEAE)	Wild tamarind, White Leadtree, Lead Tree, Koa Haole, Ekoa, Leucaena, Horse Tamarind, Jumbie Bean, White Popinac (Eng.)	Bark, leaves, seeds	Internal pain, contraceptive, ecbolic, depilatory, colds, fevers, flu, circulatory problems, to calm nerves, tuberculosis, reduce back pain and menstrual cramps. Edible seed sufficiently cooked. The seeds can be roasted, ground and used as a coffee	PMDN 343

			substitute (Duke, 1983).	
Burkea africana Hook. (LEGUMINOSAE)	Wild seringa (Eng.); wildesering (Afr.); mpulu (Tsonga), monato (Tswana); monatô (Northern Sotho); mufhulu (Venda)	Roots, bark	Tooth ache, heavy menstruation, abdominal pain, inflammation and pneumonia (Mathisen et al, 2002).	PMDN 556
Morus mesozygia (MORACEAE)	African mulberry (Eng.)	Leaves, barks, fruits, roots, stem.	Arthritis, rheumatism, etc.; malnutrition, debility; pain-killers; stomach troubles, syphilis, dermatitis, asthenias, fever and malaria (Berhaut, 1979; Burkill, 1997).	PMDN 58
Uapaca nitida (PHYLLANTHACEAE)	Narrow-leaved mahobohobo (Eng.)	Stem barks	Fever, pain, inflammation, skin diseases, sexual dysfunction (Berhaut, 1975; Kirby et al, 1993).	PMDN 88
Ziziphus rivularis Codd (RHAMNACEAE)	False buffalo thorn, river jujube (Eng.)	Roots, barks, leaves	Pains, dysentery, respiratory ailments, skin septic swellings, swollen glands, wounds and sores, snake bites (Palmer and Pitman, 1972; Hutchings et al, 1996).	PMDN 194
Englerophytum magalismontanum (Sond.) T.D.Penn. (SAPOTACEAE)	Transvaal milkplum (Eng.); stamvrug (Afr.), Motlhatswa (Tswana); Mohlatswa (Northern Sotho); Munombelo (Venda); Amanumbela (Zulu); UmNumbela (Swati)	Roots, fruits	Rheumatism, abdominal pain, epilepsy (Coates, 2002)	PMDN 600
Lippia javanica (Burm.f.) Spreng. (VERBENACEAE)	Fever tea/ Lemon Bush (Eng.) Koorsbossie Beukesbossie Lemoenbossie (Afrikaans) mutswane, umSutane (Swati) inZinziniba (Xhosa) umSuzwane, umSwazi (Zulu) musukudu, bokhukhwane (Tswana)	Leaves, twigs, roots	Coughs, colds and bronchial problems, fever, asthma, chronic coughs and pleurisy, Skin disorders. (Hutchings et al, 1996; Van Wyk, et al, 1997; Watt and Breyer-Brandwijk, 1962).	PMDN 435

(Eng.)=English, (Afr.)=Afriakaans

2.2. Chemicals

Ferric chloride and linoleic acid were purchased from Merck, Darmstadt and Schuchardt (Germany) respectively. Xylenol orange was obtained from Searle Company, England. Sodium carbonate was obtained from Holpro Analytic, South Africa. Foetal calf serum (FCS) and Dulbecco's modified Eagle's medium (DMEM) was provided by Highveld Biological, Johannesburg, South Africa. Phosphate buffered saline (PBS) and trypsin were purchased from Whitehead Scientific, South Africa. Quercetin, 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Folin-Ciocalteu reagent, gallic acid, 2,5,7,8-tetramethylchroman carboxylic acid (Trolox) and potassium persulfate were purchased from Sigma-Aldrich St. Louis, MO, USA . Sodium dodecyl sulphate, potassium ferric cyanide, 2,4,6-Tri(2-pyridyl)-s-triazine (TPTZ), HCl, iron (III) chloride ($FeCl_3$. $6H_2O$), iron (II) sulfate, tris, bovine serum albumin (BSA), sodium chloride (NaCl), $MgCl_2 \cdot 6H_2O$, acetylthiocholine iodide (ATCI), Eserine, 5,5-dithiobis-2-nitrobenzoic acid (DTNB), acetylcholinesterase (AChE) enzyme from electric eels (type VI-S lypophilized powder) and 15-lipoxygenase from *Glycine max* were provided by Sigma, Germany. Tris(hydroxymethyl)aminomethane was purchased from Sigma, Switzerland.

2.3. Anti-inflammatory activity

2.3.1. Soybean lipoxygenase inhibition assay

The assay was performed according to a previously described procedure (Pinto et al., 2007) with slight modifications. The assay is based on measuring the formation of the complex $Fe3^{+}$/xylenol orange in a spectrophotometer at 560 nm. 15-Lipoxygenase from *Glycine max* was incubated

with extracts or standard inhibitor at 25°C for 5 min. Then linoleic acid (final concentration, 140 µM) in Tris-HCl buffer (50 mM, pH 7.4) was added and the mixture was incubated at 25°C for 20 min in the dark. The assay was terminated by the addition of 100 µL of FOX reagent consisting of sulfuric acid (30 mM), xylenol orange (100 µM), iron (II) sulfate (100 µM) in methanol/water (9:1). For the control, only LOX solution and buffer were pipetted into the wells. Blanks (background) contained the enzyme LOX during incubation, but the substrate (linoleic acid) was added after the FOX reagent. The lipoxygenase inhibitory activity was evaluated by calculating the percentage of the inhibition of hydroperoxide production from the changes in absorbance values at 560 nm after 30 min at 25°C. % inhibition = $[(A_{control} - A_{blank}) - (A_{sample} - A_{blank})/ (A_{control} - A_{blank})]$ x100. Where, $A_{control}$ is the absorbance of control well, A_{blank} is the absorbance of blank well and A_{sample} is the absorbance of sample well.

2.3.2. Assay of nitric oxide production and viability of LPS-activated RAW 264.7 macrophages

Cell culture

The RAW 264.7 macrophages cell lines obtained from the American Type Culture Collection (Rockville, MD, USA) were cultured in plastic culture flask in DMEM containing L-glutamine supplemented with 10% foetal calf serum (FCS) and 1% PSF (penicillin/streptomycin/fungizone) solution under 5% CO_2 at 37 ^{0}C, and were split twice a week. Cells were seeded in 96 well-microtitre plates and were activated by incubation in medium containing LPS (5 µg/mL) and various concentrations of extracts dissolved in DMSO (final DMSO concentration of 0.2%).

Measurement of nitrite

Nitric oxide released from macrophages was assessed by the determination of nitrite concentration in culture supernatant using the Griess reagent. After 24 h incubation, 100 µL of supernatant from each well of cell culture plates was transferred into 96-well microtitre plates and equal volume of Griess reagent was added. The absorbance of the resultant solutions was determined on a BioTek Synergy microplate reader after 10 min at 550 nm. The concentrations of nitrite were calculated from regression analysis using different concentrations of sodium nitrite to deliver a standard curve. Percentage inhibition was calculated based on the ability of extracts to inhibit nitric oxide formation by cells compared with the control (cells in media without extracts containing triggering agents and DMSO), which was considered as 0% inhibition.

Cell viability

To determine that the observed nitric oxide inhibition was not due to cytotoxic effects, a cytotoxicity assay was also performed following culture as previously described by Mosmann [39], with slight modifications. After removal of media, the cells were topped up with 200 µL DMEM. To each well, 30 µL of 15 mg/mL 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetra-zoliumbromide (MTT) were added. The cells were incubated at 37 ^{0}C with 5% CO_2. After 2 h, the medium was carefully discarded and the formed formazan salt was dissolved in DMSO. The absorbance was read at 570 nm (SpectraMax 190, Molecular devices). The percentage of cell viability was calculated with reference to the control (cells without extracts containing LPS taken as 100% viability). All the experiments for the measurement of nitric oxide inhibition were conducted three times in triplicate.

2.4. Acetylcholinesterase inhibition assay

Inhibition of acetylcholinesterase activity was determined using Ellman's colorimetric method (1961) with some modifications. In a 96-well plate was placed: 25 µL of 15 mmol/L ATCI in water, 125 µL of 3 mmol/L DTNB in Buffer A (50 mmol/L Tris-HCl, pH 8, containing 0.1 mol/L NaCl and 0.02 mol/L $MgCl_2.6H_2O$), 50 µL of Buffer B (50 mmol/L, pH 8, containing 0.1 % bovine serum albumin) and 25 µL of plant extract (0.007 mg/mL, 0.016 mg/mL, 0.031 mg/mL, 0.063 mg/mL or 0.125 mg/mL). Then, AChE (0.2 U/mL) was added to the wells and the absorbance was determined spectrophotometrically (BioTek Synergy microplate reader) at 405 nm. Eserine and water were used as the positive and negative controls respectively. Percentage inhibition was calculated by comparing the reaction rates for the sample to the negative control using the following formula: $[(A_{control} - A_{blank}) - (A_{sample} - A_{blank})/ (A_{control} - A_{blank})]$ x100. Where, $A_{control}$ is the absorbance of control well, A_{blank} is the absorbance of blank well and A_{sample} is the absorbance of sample well. Results are presented as means ± standard errors of the experiment in triplicate. The IC_{50} values of plant extracts showing percentage inhibition >50% were calculated by plotting the percentage inhibition against extract concentration.

2.5. Phytochemical analysis

2.5.1. Total phenolic content (TPC) determination

The total phenolic content of extracts was determined colorimetrically using a 96-well microplate Folin-Ciocalteu assay developed by Zhang et al. (2006). The total phenolic content was calculated from the linear equation of a standard curve prepared with gallic acid, and expressed as gallic acid equivalent (GAE) per g of extract.

2.5.2. Total flavonoids content (TFC) determination

Total flavonoid content was determined using the method of (Ordonez et al., 2006). A volume of 0.5 mL of 2% $AlCl_3$ ethanol solution was added to 0.5 mL of sample solution (1 mg/mL). After one hour at room temperature, the absorbance was measured at 420 nm. A yellow color is indicative of the presence of flavonoids. Total flavonoid content was calculated and expressed as mg quercetin equivalent/g of crude extract using a standard curve prepared with quercetin.

2.6. Antioxidative activity

2.6.1. ABTS radical assay

The ABTS radical scavenging capacity of the samples was measured with modifications of the 96-well microtitre plate method described by Re et al. (1999). Trolox and ascorbic acid were used as positive controls, methanol as negative control and extract without ABTS as blank. The percentage of ABTS•+ inhibition was calculated using the formula: Scavenging capacity (%) = 100 - [(absorbance of sample - absorbance of sample blank) × 100/ (absorbance of control) – (absorbance of control blank)]. The IC_{50} values were calculated from the graph plotted as inhibition percentage against the concentration.

2.6.2. DPPH assay

The DPPH radical-scavenging activity was determined using the method proposed by Brand-Williams (1995). Ascorbic acid and trolox were used as positive controls, methanol as negative control and extract without DPPH as blank. Results were expressed as percentage reduction of the initial DPPH absorption in relation to the control. The concentration of extract that reduced the DPPH colour by 50% (IC_{50}) was determined using ABTS•+.

2.6.3. Ferric Reducing Antioxidant Power (FRAP) assay

The FRAP assay was carried out according to the procedure of Benzie and Strain (1996) with slight modifications. Briefly, the FRAP reagent was prepared from acetate buffer (pH 3.6), 10 mmol TPTZ solution in 4 0 mmol HCl and 20 mmol iron (III) chloride solution in proportions of 10:1: 1 (v/v), respectively. The FRAP reagent was prepared fresh daily and was warmed to 37º C in a water bath prior to use. Fifty microliters of sample were added to 1.5 ml of the FRAP reagent. The absorbance of the reaction mixture was then recorded at 593 nm after 4 min. The standard curve was constructed using $FeSO_4$ solution (0.1-2 mM), and the results were expressed as µmol $FeSO_4$/g dry weight of crude extract. All the measurements were taken in triplicate and the mean values were calculated.

2.7. Statistical analysis

All experiments were conducted in triplicate and values expressed as mean ± standard deviation. Differences between values were assessed for significance using analysis of variance and results were compared using the Fisher's least significant difference (LSD) at a 5% significance level.

3. Results

3.1. Anti-inflammatory activity

The 15-lipoxygenase inhibiting activity was measured using the 96-well microplate-based ferric oxidation of xylenol orange (FOX) assay. In a preliminary screening to select samples for dose-response study, extracts and the reference compounds (quercetin and indomethacin) were tested at a single concentration of 100 µg/mL. The results summarized in Fig. 1A revealed that, extract of *Burkea africana* had the highest anti-15-lipoxygenase activity with 85% inhibition while

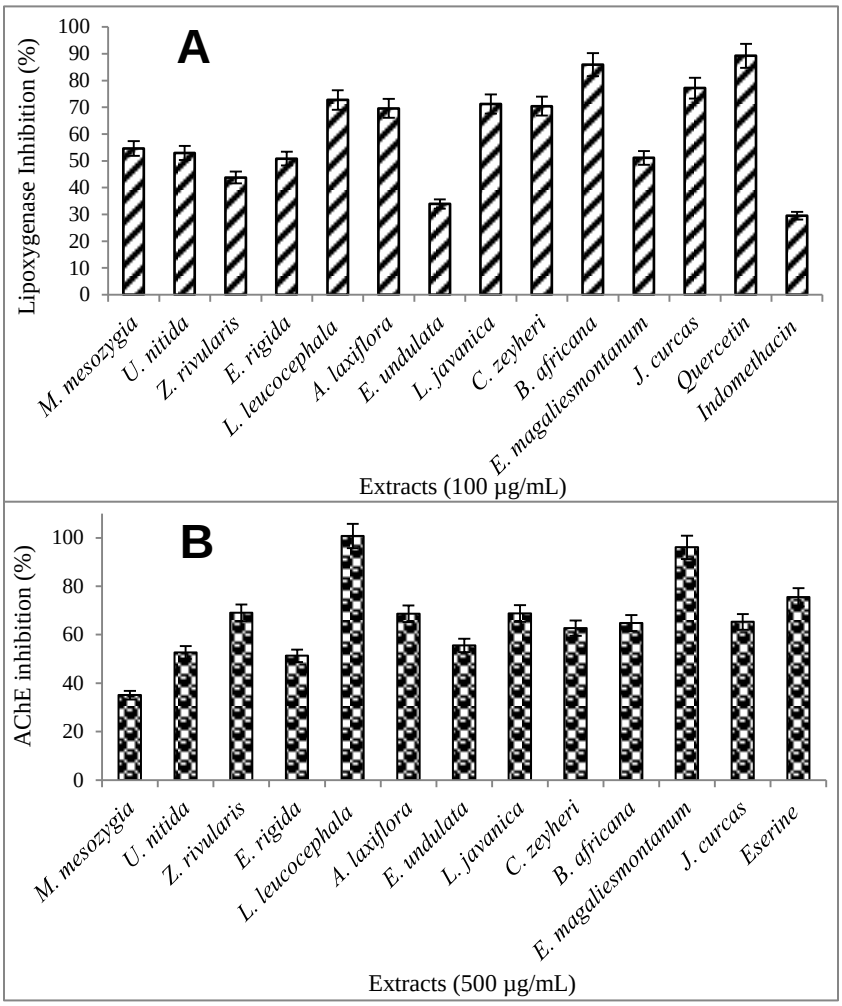


Figure 1: Percentage inhibition of LOX (A) and AChE (B) of the twelve South African medicinal plants traditionally used to alleviate pain and inflammations tested at 100 µg/mL in LOX assay and 500 µg/mL in AChE assay.

Euclea undulata had the lowest activity (33.88%). According to the classification LOX inhibition activity by plant extracts suggested by Chung, et al, (2009) apart from the extract of *Euclea undulata* (33.88%), all the extracts had high to moderate activity against 15-lipoxygenase. Extracts with LOX inhibitory activity greater than 50% in a preliminary screening were then tested between 0-128µg/mL in the confirmation assay and they inhibited LOX in a

concentration dependent manner (Table 2). The IC_{50} values ranged between 37.25 µg/mL (*Burkea africana*) and 63.25 µg/mL (*Englerophytum magaliesmontanum*).

Table 2: Estimated IC_{50} (µg/ml) for 15-lipoxygenase (LOX) and acetylcholinesterase (AChE) of the twelve South African medicinal plants traditionally used to alleviate pains and inflammations.

Plant name	**Yield*** **(%)**	**15-LOX IC_{50} (µg/mL)**	**AChE IC_{50} (µg/mL)**
M. mesozygia	5.56	58.62 ± 0.15^{a}	-
U. nitida	1.13	60.50 ± 3.13^{a}	$475.12\pm9.12^{a,b}$
Z. rivularis	4.23	-	$362.52\pm12.89^{c,d}$
E. rigida	4.70	62.99 ± 1.43^{a}	487.44 ± 0.17^{a}
L. leucocephala	4.43	43.99 ± 0.79^{c}	118.23 ± 4.40^{e}
A. laxiflora	2.33	46.03 ± 2.10^{b}	364.12 ± 2.39^{f}
E. undulata	8.63	-	$450.34\pm18.71^{b,g}$
L. javanica	3.70	$44.99\pm2.37^{b,c}$	$363.74\pm11.16^{c,h}$
C. zeyheri	8.50	45.46 ± 0.75^{b}	398.62 ± 2.41^{i}
B. africana	13.5	37.25 ± 0.88^{e}	$386.11\pm16.66^{i,c}$
E. magaliesmontanum	9.56	63.25 ± 9.11^{a}	160.19 ± 1.81^{j}
J. curcas	3.96	$41.50\pm1.24^{b,d}$	382.89 ± 2.01^{c}
Quercetin		35.85 ± 0.18^{f}	nd
Indomethacin		-	nd
Eserine		nd	4.94 ± 0.05^{k}

nd: not determine; *: extraction yield; -: <50%inhibition;

Values with different letters are significantly different at $p< 0.05$.

The NO production inhibitory activity of plant extracts were evaluated in LPS-activated malignant macrophages cell line RAW264.7. Validity of the assays was shown by using untreated cells as negative control, LPS-stimulated cells as positive control and additionally a cell group as reduction control group with LPS-stimulated cells, co-incubated together with quercetin used as an inhibitor of NO (Mu et al, 2001). As shown in Table 3, all the extracts showed dose dependent inhibition of NO production at the concentration of 6.25, 12.5, 25 and 50µg/mL. At the highest concentration (50 µg/mL), all the extracts evaluated had percentage inhibition greater than 90%. *L. leucocephala* and *L. javanica* had the most potent inhibition with percentage inhibition of 97.52% and 97.40% respectively at the concentration of 25 µg/mL and cell viability of 86.52% and 85.52% respectively.

Table 3: Inhibitory activities of the twelve South African medicinal plants traditionally used to alleviate pains and inflammations on nitric oxide production and viability of LPS-activated RAW 264.7 macrophages.

Plant name	Concentration (µg/mL)	NO (µM)	% NO inhibition	% Cell viability
	50	0.36±0.16	95.42	61.07
Morus	25	0.57±0.31	92.69	67.83
mesozygia	12.5	1.16±0.37	85.14	67.60
	6.25	2.86±0.40	63.22	79.00
	50	0.30±0.01	96.16	57.90
Uapaca	25	0.48±0.09	93.81	66.38
nitida	12.5	1.55±0.85	80.06	76.81
	6.25	3.15±1.07	59.51	75.90
	50	0.39±0.08	95.05	71.67
Ziziphus	25	0.60±0.08	92.32	71.71
rivularis	12.5	1.29±0.28	83.41	72.24
	6.25	3.87±1.49	50.22	78.50
	50	0.64±0.36	91.83	34.26
Ehretia	25	0.58±0.12	92.57	55.05
rigida	12.5	0.78±0.26	89.97	58.98

	6.25	1.07±0.50	86.25	63.86
	50	0.34±0.07	95.67	76.24
Leucaena	25	0.19±0.11	97.52	86.52
leucocephala	12.5	0.39±0.16	94.92	71.17
	6.25	0.73±0.03	90.59	65.12
	50	0.48±0.03	93.81	58.93
Alchornea laxiflora	25	0.27±0.03	96.53	62.50
	12.5	0.70±0.23	90.96	61.36
	6.25	1.06±0.06	86.38	63.83
	50	0.73±0.13	90.59	84.95
Euclea	25	0.56±0.19	92.82	80.71
undulata	12.5	0.99±0.26	87.25	80.79
	6.25	2.30±0.63	70.40	75.12
	50	0.34±0.09	95.67	56.29
Lippia	25	0.20±0.15	97.40	56.79
javanica	12.5	1.09±0.41	86.01	64.38
	6.25	2.94±0.73	62.23	85.52
	50	0.26±0.02	96.66	55.98
Combretum	25	0.31±0.03	96.04	61.60
zeyheri	12.5	1.06±0.30	86.38	67.17
	6.25	2.67±0.54	65.70	63.79
	50	0.62±0.21	92.07	53.76
Burkea	25	0.67±0.19	91.33	66.14
africana	12.5	1.13±0.05	85.51	71.83
	6.25	3.05±0.38	60.75	71.88
	50	0.44±0.06	94.30	98.26
Englerophytum.	25	0.29±0.02	96.29	99.60
magaliesmontanum	12.5	0.64±0.11	91.83	77.71
	6.25	0.85±0.13	89.10	72.02
	50	0.57±0.20	92.69	71.45
Jatropha	25	0.31±0.08	96.04	71.60
curcas	12.5	0.53±0.06	93.19	75.64
	6.25	1.17±0.19	85.02	77.79
	25	0.35±0.10	95.54	49.33
Quercetin	12.5	0.30±0.08	96.16	60.69
	6.25	0.69±0.05	91.08	73.76
	3.12	2.50±0.48	67.93	73.10

3.2. Acetylcholinesterase inhibition assay

Extracts were tested in a preliminary assay at a single concentration of 500 µg/mL compared to eserine (standard AChE inhibitor) at a concentration of 10 µg/mL. At a concentration of 500 µg/mL, the extract from *L. leucocephala* had the highest AChE inhibitory activity (with 100.73% inhibition) followed by *E. magalismontanum* (96.08%). *Morus mesozygia* had the lowest AChE inhibitory activity with 35.05% inhibition. Apart from the extract of *M. mesozygia*, all the extracts were active enough to determine the IC_{50} values (Table 2). All the extracts evaluated had dose-dependent inhibition. Eserine used as a positive control AChE inhibitor in this study inhibited 50% of AChE activity (IC_{50}) at a concentration of 4.94±0.05 µg/mL.

3.3. Antioxidant activity and phytochemical analysis

Extracts were tested for their antioxidant potential using three different methods including the DPPH, the ABTS and the FRAP assays. The highest antioxidant activity was demonstrated by *C. zeyheri* (IC_{50} values of 3.52±0.46 µg/mL and 4.64±0.48 µg/mL in DPPH and ABTS assays respectively) and *B. africana* (IC_{50} of 3.55±0.32 µg/mL and 3.12±0.54 µg/mL in DPPH in ABTS respectively). In DPPH assay, no significant difference was observed between the antioxidant capacity of the two plants species as compare to standard antioxidant trolox and ascorbic acid. *M. mesozygia*, *E. magaliesmontanum*, *L. leucocephala* and *L. javanica* had moderate antioxidant activity (IC_{50} varying from 7.98±0.84 to 18.53±1.42 µg/mL) whereas the other remaining extracts had comparatively low activity (IC_{50} varying from 28±2 to 235±3 µg/mL). The IC_{50} values in DPPH were close to values obtained in the ABTS analysis, probably due to the similarity of both methods involving one electron-transfer. FRAP is the ferric reducing power of antioxidants by the reduction of the ferric ions to the ferrous ions. The results expressed as l µg

ferrous iron equivalents per g of crude extract are shown in Table 4. The IC_{50} values ranged from 76.00±3.21 to 578±32 µg Fe (II)/g.

Table 4: Antioxidant activity, total phenolics and flavonoids content of twelve South African medicinal plants traditionally used to alleviate pains and inflammations.

Plant name	DPPH IC_{50} (µg/mL)	ABTS IC_{50} (µg/mL)	FRAP IC_{50} (µg Fe (II)/g)	TPC (mg GAE/g)	TFC (mg QE/g)
E. rigida	171.75±3.46^{a}	234.83±3.18^{a}	94.07±3.26^{a}	103.28±4.26^{a,b}	6.34±2.77^{a}
J. curcas	137.08±4.03^{b}	115.23±2.66^{b}	68.17±4.44^{b}	130.12±17.06^{a}	60.87±0.54^{a}
U. nitida	125.86±3.85^{c}	28.81±2.45^{c}	177.32±12.55^{c}	137.30±46.40^{c,d}	15.37±0.37^{a}
Z. rivularis	74.05±4.80^{d}	36.52±1.57^{d}	286.31±67.93^{d,e}	182.79±27.97^{c,d}	46.88±10.41^{b}
E. undulata	31.66±0.58^{e}	32.67±0.45^{e}	274.19±29.36^{d}	129.78±32.58^{a,b}	35.16±2.49^{b}
A. laxiflora	17.19±1.02^{f}	18.53±1.42^{f}	438.42±15.55^{f}	147.95±30.18^{c,d}	13.84±1.47^{b}
M. mesozygia	15.85±0.81^{f,g}	271.86±6.43^{g}	127.34±18.34^{g}	181.49±13.12 ^{e}	13.75±2.44^{c}
E. magaliesmontanum	10.80±0.56$_{h}$	12.22±3.24^{h}	76.00±3.21^{h}	100.89±4.00^{c}	68.43±2.04^{d,a}
L. leucocephala	9.86±0.59^{i,j}	9.05±0.81^{h,i}	289.25±82.87^{d}	258.40±7.45 ^{c,d,e}	159.61±12.79^{a,e}
L. javanica	7.98±0.84^{k}	18.18±1.50^{f,j}	579.49±32.21^{i}	427.53±88.63^{e,f}	80.72±4.93^{a}
B. africana	3.55±0.32^{l}	3.12±0.54^{k}	231.07±91.43^{d,e,j}	150.82±17.43^{g}	72.80±21.42^{d}
C. zeyheri	3.52±0.46^{l}	4.64±0.48^{l}	95.98±5.30^{a}	234.56±63.70^{a}	64.36±5.86^{f}
Trolox	3.14±0.10^{l}	6.05±0.24^{l}	nd	nd	nd
Ascorbic acid	1.95±0.04^{l}	3.57±0.18^{k}	nd	nd	nd

Values with different letters are significantly different at $p< 0.05$.

As depicted in Table 2, all the plant species invested found to be rich in phenolic compounds with values ranged from 428±88 to 100±4mg GAE/g. Similarly, all the plant species tested had significantly higher total flavonoids content with values ranged from159±12 to 6±2 mg QE/g.

The highest phenolics content was observed in extract s of *L. leucocephala*, *L. javanica*, *C. zeyheri*. Negative correlation was found between the antioxidant activities and the total phenolic and flavonoids content (Table 5).

Table 5: Pearson's correlation coefficients of the antioxidant activity (DPPH, ABTS, FRAP) and the total polyphenol content (TPC) and total flavonoid (TFC) of extracts from of twelve South African medicinal plants traditionally used to alleviate pains and inflammations. No significant relationship was found between pairs of variables in the correlation ($p > 0{,}050$).

		TPC	TFC
DPPH	r^2	-0,437	-0,441
	p	0,155	0,152
ABTS	r^2	-0,257	-0,498
	p	0,420	0,0994
FRAP	r^2	-0,279	-0,292
	p	0,380	0,357
TPC	r^2		0,490
	p		0,106

r: correlation coefficient, p: p value

4. Discussion

All the plants investigated are traditionally used to alleviate pain and inflammation in South Africa. Most of the plants are indigenous to South Africa. The anti-inflammatory properties of these plants were evaluated on the basis of their ability to inhibit the 15-lipoxygenase as well as their NO production inhibiting effect in LPS activated RAW 264.7 macrophages. The 15-lipoxygenase enzyme is the key in leukotriene biosynthesis and catalyses the initial steps in the

conversion of arachidonic acid to biologically active leukotrienes. Leukotrienes are considered as potent mediators of inflammatory and allergic reactions and regarding their pro-inflammatory properties the inhibition of 15-lipoxygenase pathway is considered to be interesting in the treatment of a variety of inflammatory diseases (Schneider and Bucar, 2005). The IC_{50} values of extracts obtained in 15-LOX inhibitory activity are similar to those of other LOX active crude extracts in the range of 1-100µg/mL (Schneider and Bucar, 2005). Particularly *Burkea africana* with most potent activity (37.25±0.88 µg/mL) could be regarded as a potential source for new 15-LOX inhibitors. Hydroethanol extract from the bark of *Burkea africana* has been reported to exhibit lipoxygenase inhibitory activity (IC_{50} of 37 µg/mL), and the inhibiting effect is due to the presence of profisetinidin-type proanthocyanidins (IC_{50} of 21 µg/mL) (Mathisen et al, 2002). It is noteworthy that indomethacin had insignificant LOX inhibitory activity as expected. Although used as a non-steroidal anti-inflammatory drug, indomethacin is a nonselective inhibitor of cyclooxygenase 1 and 2 (Hart and Boardman, 1963).

In NO inhibitory activity assay, four different concentrations (6.25, 12.5, 25 and 50 µg/mL) of the extracts were used and the cellular viability was also determined. RAW 264.7 cells were incubated with extracts and cell viability was measured by an MTT assay. We found that extract had no cytotoxic effects on RAW 264.7 cells at the highest concentration (50 µg/mL) tested. This result confirmed that the effects of extracts on RAW 264.7 cells were not due to their cytotoxicity. The anti-inflammatory activity of the studied plants was confirmed by inhibition of NO production (Table 3). In inflammation, nitric oxide (NO) acts as a pro-inflammatory mediator and is synthesized by inducible nitric oxide synthase (iNOS) in response to pro-inflammatory agents such as lipopolysaccharide (LPS). (Lu et al, 2008). The inhibitory activity of NO production by medicinal plants may come from the inhibition of iNOS enzyme activity

and/or expression of nitric oxide synthase (Ryu et al, 2003). Many natural compounds from medicinal plants have been known as inhibitors of expression of iNOS in LPS-activated macrophages. (Son et al, 2000). As far as we know, no report in the literature was found on the inhibitory activity of plant extracts on NO synthesis in LPS-activated RAW 264.7 cells.

All the extracts evaluated inhibited AChE to some extent. Antiinflammatory activity of plant extract at 250 µg/ml above 70% is considered significant (Chinsamy et al, 2014), considering this cut-off, only two extracts (*L. leucocephala* and *E. magaliesmontanum*) were potent in AChE assay. Medicinal plants have been previously reported as having acetylcholinesterase inhibitor ability (Chinsamy et al, 2014, Mathew and Subramanian, 2014) and a number of references reported the main components as phenolics and alkaloids (Adewusi et al, 2012; Aderogba et al, 2013). However, the AChE inhibitory activities of the plant species we studied have never been reported before. It may be interesting to determine the alkaloid content of extracts with high anticholinesterase activity in a follow up study because several compounds with anticholinesterase activity are alkaloids (Elisha et al., 2013). It is possible that some false positive results in the Ellman assays used were obtained. Rhee et al. (2003) investigated the inhibition of the acetylcholinesterase by plant extracts directly on bioautograms and found that many aldehydes and amines that could be present in plant extracts give false positive reactions because they do not inhibit the enzyme but inhibit the reaction between thiocholine and 2-nitrobenzoic acid. The detection limit was about a thousand times higher than that of galanthamine. They proposed that before isolating bioactive compounds false positives should be detected by spraying duplicate chromatograms with relevant spray reagents that would detect aldehydes and amines and comparing R_f values. If the ratio of 1000 to 1 between true and false positives is also correct in the assay we used, it is unlikely that the EC_{50} values we found were

due to false positives because the ratios were not higher than 100 times compared to eserine (Table 2).

A wide variety of methods have been used to determine the antioxidant activity of samples and no single assay provides an accurate method to determine the capacity to scavenge free radicals and/or to prevent lipid oxidation, particularly in a mixed or complex system such as plant extracts. Therefore, it is essential to use diverse methods to assess different aspects of the oxidation process. Furthermore, different compounds may act as antioxidants through different mechanisms. Therefore, it has been recommended that at least two different assays should be used in evaluating antioxidant activity of plant (Moon and Shibamoto, 2009). Consequently, extracts were tested for their antioxidant potential using the DPPH, the ABTS and the FRAP methods. Among the twelve plants species investigated, two medicinal plants which are potentially rich sources of natural antioxidants were identified: *C. zeyheri* and *B. africana*. Qualitative antioxidant activity has been found in the methanolic and acetone extract of the leaves of *C. zeyheri*. (Masoko and Eloff, 2007). Antioxidant activity of aqueous methanol leaf extract of *Leucaena leucocephala* was previously reported with the isolation of flavonoids quercetin-3-O-arabinofuranoside, quercetin-3-O-rhamnoside and apigenin as main antioxidant constituents (Aderogba et al, 2009). As far as we know, this is the first report in the literature of the antioxidant activity of extract from *L. javanica*. Discrepancy in the antioxidant activity values depending on the method used indicates that each method determine different aspects of the antioxidant capacity. In fact, different radicals and mechanisms of reaction are occurring since FRAP assay involves single electron transfer (SET) method, while DPPH and ABTS assay involves both single electron transfer (SET) and hydrogen atom transfer (HAT) methods predominantly via SET method (Badarinath et al, 2010).

Phenolic compounds have been shown to be responsible for the antioxidant activity of plant materials (Rice-Evans et al, 1996). Therefore, the total phenolic content in plant extracts was investigated. Many studies have focused on the biological activities of phenolics which are potent antioxidants and free radical scavengers, the antioxidant activity of phenolics including flavonoids being mainly due to their redox properties, which allow them to act as reducing agents, hydrogen donors, and singlet oxygen quenchers (Rice-Evans et al, 1996). No good correlation was found between the antioxidant activities and the total phenolic and flavonoids content. The antioxidant activity of extracts could therefore not only be explained on the basis of their phenolic content, but required also their proper characterization. It is known that only flavonoids with a certain structure and particularly hydroxyl position in the molecule can act as proton donating and show radical scavenging activity (Nickavar et al, 2007; Wojdylo et al, 2007). Furthermore, the extracts are very complex mixtures of many different compounds with distinct activities. The lack of relationship observed in this study is in agreement with other report in the literature (Ghasemi et al, 2009). Sengul et al. (2009), also reported a negative correlation between total phenolic content and antioxidant capacities of a number of medicinal plant extracts.

Although all the investigated plants are traditionally used against inflammatory conditions, some did not show significant activity in the current study. However, weak activity obtained suggested that active compound(s) may be present in insufficient quantities in the crude extracts to show strong activity with the dose levels used (Taylor et al., 2001). It is possible that other mechanism to control pain that we have not investigated or a placebo effect may be involved.

The overall results obtained from this study indicate that some of the plants species investigated have a potential to be used as an antioxidant, anti-inflammatory and anti-cholinesterase agent.

These findings are consistent with those found in the literature, since plant phenolics are well-known for their potent antioxidant activities. Some phenolic compounds like quercetin have an anticholinesterase activity (Ji and Zhang, 2006). Furthermore, phenolic compounds have been reported to be beneficial in the treatment of chronic inflammatory diseases associated with overproduction of nitric oxide (NO) (Jiang and Dusting, 2003).

Conclusion

The primary objective of this study was to determine if extracts of the twelve species used traditionally to alleviate pains and inflammations have *in vitro* anti-inflammatory activity. In most cases the *in vitro* results support the traditional use. Because antioxidant compounds may also be active in neurogenerative diseases, the anticholinesterase activity of extracts was also determined. Extracts of *Burkea africana, Leucaena leucocephala*, and *Lippia javanica* could be regarded as promising sources of interesting 15-LOX, NO production and AChE inhibitors as well as antioxidants. The bioactive compounds should be isolated and its safety should be determined to investigate potential use. Animal studies are also required to determine if *in vitro* activity of extracts equates to *in vivo* activity.

Acknowledgments

The University of Pretoria provided a postdoctoral fellowship to JPD. The National Research Foundation (NRF) and Medical Research Council (MRC) provided funding to support this study.

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