Anti-inflammatory, antioxidant, anti-cholinesterase activity and mutagenicity of South African medicinal orchids

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While the role of various processes in inflammatory-related degenerative disorders is still being researched, many avenues of research have concentrated on the treatment and/or prevention of these disorders. Inflammatory-responses, the cholinergic system and oxidative stress have often been linked to the symptoms prevalent in aged persons and Alzheimer’s patients. The current research explored the selective inhibition of cyclooxygenase (COX) enzymes, antioxidant and anti-cholinesterase activities of selected South African orchid extracts, currently traded in herbal markets along the east coast of South Africa. Out of a total of 53 evaluated extracts, significant anti-inflammatory activity was observed in nearly 40% of extracts in the COX-1 assay and 25% of extracts in the COX-2 assay. Overall, the DCM root extract of Ansellia africana was the most potent, the DCM tuber extract of Eulophia hereroensis was the only extract to significantly inhibit both COX enzymes, while all Bulbophyllum sabeurilum organic root extracts exhibited COX-2 selective inhibitory activity. Bulbophyllum sabeurilum DCM root extract was also the most effective anti-cholinesterase extract, performing better than galanthamine. In the single electron transfer (SET) (2,2’-diphenylpicrylhydrazyl (DPPH) free radical scavenging assay and ferric reducing antioxidant power (FRAP) reaction based assays, E. petersii pseudobulb and A. africana root extracts performed better than other extracts. In the hydrogen atom transfer (HAT) (•-carotene/linoleic acid assay) reaction-based assay, the leaf extract of Tridactyle tridentata and root extracts of Cyrtorchis arcuata and E. hereroensis exhibited the best antioxidant effects. The mutagenicity (Salmonella/microsome assay) was also determined. Organic leaf and root extracts of C. arcuata produced no genotoxic effects in comparison to the other tested species. None of the crude extracts tested demonstrated mutagenic effects using S. typhimurium strain TA98 with metabolic activation. The results obtained in this study validate the use of certain orchid species in South African traditional medicine for inflammation-related degenerative disorders.

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1. Introduction

Since the detection of prostaglandins (PGs) in the brain, the function, distribution and expression of all constituents including cyclooxygenase (COX) enzymes in the inflammatory process have been researched (Kaufmann et al., 1997). While a more comprehensive understanding is needed, the role of anti-inflammatory agents in the prevention or treatment of Alzheimer’s has been the focus of current research (Moore and O’Banion, 2002). According to Rich et al. (1995) and DeKosky (2003) certain non-steroidal anti-inflammatory drugs (NSAIDs) when administered over a long time exhibited a decreased risk of Alzheimer’s. The varying effects of different classes of anti-inflammatory agents in different trials suggest the use of anti-inflammatory agents as a preventative measure and not as a treatment (DeKosky, 2003). Montine et al. (1999) demonstrated that while COX activity in persons susceptible to Alzheimer’s was comparable to that of control persons; concentration levels of PGE2 in cerebrospinal fluid of susceptible patients had increased five times. Their study demonstrated the potential of COX-inhibitors in the treatment of Alzheimer’s. By selectively inhibiting the inducible COX-2, one could possibly produce an efficient and tolerable anti-inflammatory regime that could be used to treat symptoms of common inflammatory disorders such as pain and fever; and that could be used to prevent the onset and/or treat symptoms of diseases affecting the CNS.

According to Borovikova et al. (2000) there is an established link between the cholinergic system and inflammation, with acetylcholine (ACH) playing a role in cytokine release. Tabet (2006) reported on evidence that acetylcholinesterase (ACHE) inhibitors have an anti-inflammatory role by indirectly increasing the production of antioxidants in the brain, thereby acting against free radicals, amyloid toxicity...
and a reduction in release of cytokines from activated microglia in the brain and blood. Oxidative stress has been associated with several degenerative disorders; which include cancer, arteriosclerosis, inflammatory disorders and neurodegenerative diseases. Processes such as lipid peroxidation have been implicated in neurodegenerative diseases such as Alzheimer's (Markesbury and Carney, 1999). The ageing process has also been associated with oxidative stress (Markesbury and Carney, 1999). Tabet (2006) also suggested that the anti-inflammatory action of antioxidant compounds has a positive effect in Alzheimer's patients. Selkoe (2005) suggested the use of antioxidants and free radical scavengers as possible treatment options for certain features of Alzheimer's.

Natural plant products have been used as poultices and/or anti-inflammatories and as antioxidants for years. Bohlin (1995) listed flavonoids, naphthoquinones, alkylamides, phenolic phenyl-propane derivatives, among others, as those compounds responsible for COX inhibition in certain natural products. In the Southern African region approximately 494 orchid species occupy restricted distribution ranges, with 75% endemism. From the literature, it has been ascertained that approximately 14 South African orchid species from 7 genera are used for medicinal purposes in the practice of South African traditional medicine (Chinsamy et al., 2011). Following the review which revealed the pharmacological potential for South African medicinal orchid species, this research article demonstrates the mutagenicity and validity of medicinal use of certain South African orchid species for inflammatory-related degenerative disorders.

2. Materials and methods

2.1. Plant selection and extract preparation

Various plant parts of seven indigenous orchid species; Ansellia africana Lindl. (MC 01NU), Bulbophyllum scabrumulum (Rolfe) Bolus (MC 02NU), Cytorrhis arcuata (Lindl.) Schltr. (MC 03NU), Eulophia heteroerosea Schltr. (MC 04NU), Eulophia Petersii (Rchb.f.) Rchb.f. (MC 05NU), Polystachya pubescens (Lindl.) Rchb.f. (MC 06NU) and Tridactyle tridentata (Harv.) Schltr. (MC 07NU) were purchased from herbal markets in Nongoma, Pietermaritzburg and Umlazi (KwaZulu-Natal, South Africa). Voucher specimens were deposited at the University of KwaZulu-Natal Herbarium. All plant material was dried at 37 °C for 72 h, milled into fine powders through a 1 mm ring sieve (Ultra-Centrifugal Mill ZM 200, Retsch®, Germany) and stored in airtight containers in the dark at room temperature. For extraction solvents; petroleum ether (PE), dichloromethane (DCM), ethanol (EtOH) and water were used in a sequential extraction process. In order of increasing polarity, 5 g powdered plant material were extracted in 100–200 ml cold solvent, sonicated for 1 h in an ultrasonic water bath (Julabo GMBH, West Germany), left to saturate overnight, and then filtered under vacuum using Whatman No. 1 filter paper. Resultant organic and aqueous extracts were concentrated using a rotary evaporator (Büchi, Germany) at 30 °C and a freeze-drier respectively.

2.2. Anti-inflammatory evaluation

An enzyme-based cyclooxygenase assay (COX-1 and COX-2) as described by Jäger et al. (1996) and Eldeen and Van Staden (2008) was performed. Plant extracts were tested at a concentration of 250 μg/ml per test solution. Indomethacin (Sigma-Aldrich, USA) (5 μM for COX-1 and 200 μM for COX-2) served as a positive control, while background samples with inactivated enzymes before adding [14C] arachidonic acid (16 mCi/mmol; 3.0 μM) and a solvent blank (EtOH) served as negative controls. Each assay was repeated three times with a duplicate set of samples per assay. The anti-inflammatory effect of the plant extracts, that is; percentage inhibition of prostaglandin synthesis, was determined by comparing the amounts of radioactivity (disintegrations per minute (DPM)) in a solvent blank to those of the samples. Inhibition was indicated by a decline in PGE2 formation. This was calculated using the following formula:

\[
\text{Cyclooxygenase Inhibition(%) = } \left(1 - \frac{\text{DPM}_{\text{solvent blank}} - \text{DPM}_{\text{background}}}{\text{DPM}_{\text{sample}} - \text{DPM}_{\text{background}}} \right) \times 100
\]

where DPM_{sample}, DPM_{background} and DPM_{solvent blank} is the radioactivity for plant extracts, inactivated enzyme sample and EtOH sample respectively. The EC_{50} (mg/ml) of extracts considered significantly active were also determined using extracts within a range of 250–4625 μg/ml using a two-fold serial dilution.

2.3. Acetylcholinesterase (AChE) enzyme inhibitory activity

2.3.1. Reagents for the AChE assay

Acetylcholinesterase enzyme activity was measured by spectrophotometric observation of the increase in a yellow colour produced from thiocholine when it reacts with the dithiobisnitrobenzoate ion. Three buffers (Buffer A: 50 mM Tris–HCl, pH 8; Buffer B: 0.5 g (0.1%) bovine serum albumin (BSA) in 500 ml Buffer A; Buffer C: 2.92 g NaCl and 2.03 g MgCl$_2$·6H$_2$O in 500 ml Buffer A) were prepared with Millipore water. The substrate acetylthiocholine iodide (ATCI), 5,5'–dithiobis-(2-nitrobenzoic acid) (DTNB) or Ellman's reagent, enzyme AChE (isolated from electric eel- type VI-S lypophilized powder) and galanthamine were purchased from Sigma-Aldrich.

2.3.2. The microplate assay for acetylcholinesterase inhibition

The capacity of crude orchid extracts to inhibit AChE enzyme was determined using the protocol outlined in Ellman et al. (1961). Using a 96-well microtitre plate on ice, samples (25 μl, 10 mg/ml) and the solvent blank methanol were added in triplicate to the last well and serially diluted in a 2-fold dilution up the plate. The effective concentration of samples ranged between 0.03 and 1.0 mg/ml. Positive control, galanthamine (20 μM, 50 μl) was also prepared in a 2-fold serial dilution up the plate. To this 25 μl of substrate ATCI (15 mM), 125 μl of DTNB Ellman's reagent (3 mM in Buffer C) and 50 μl of Buffer B were added in sequence to all wells. The absorbance of the reaction mixture was read at 405 nm every 45 s using an ELISA microplate reader (Opsys MR™, Dynex Technologies Inc.). Each plate was read three times to obtain a stable background or baseline value, after which, 25 μl of AChE enzyme (0.2 U/ml in Buffer A) was added to each well. The absorbance was read once again at 405 nm at 45 s intervals, five times. The effect of spontaneous hydrolysis of the substrate was corrected by subtracting the rate of reaction before adding the enzyme from the rate after the addition. Percentage inhibition of the enzyme AChE by each sample was calculated using the formula:

\[
\text{Inhibition(%) = } \left(1 - \frac{\text{Reaction rate}_{\text{sample}}}{\text{Reaction rate}_{\text{blank}}} \right) \times 100
\]

2.4. Antioxidant evaluation

The free radical scavenging antioxidant activity of crude orchid extracts were determined using the protocol outlined in Karioti et al. (2004) with slight modifications. The 2,2'-diphenylylpiryldrazyl (DPPH) free radical scavenging assay is based on a single electron transfer (SET) reaction-based assay. The DPPH radical used in this assay is a substrate that can be reduced to DPPH-H by antioxidants that have the ability to donate a hydrogen atom to the DPPH radical. Methanolic plant samples were prepared at different concentrations (0.065–50 mg/ml). Each sample (15 μl) was diluted with 735 μl methanol, to which 750 μl of a methanolic DPPH solution (0.1 mM) (Sharma and
In the assay was 50 µM. A separate set of samples, referred to as correction factor solutions, were prepared without the methanolic DPPH solution (DPPH solution was substituted with methanol). Two antioxidants, ascorbic acid and BHT, were prepared as the positive controls (5–80 µM). Methanol served as the negative control. All reaction vessels were incubated for 30 min at room temperature in the dark. The absorbance of each sample was measured spectrophotometrically at 517 nm (Varian Cary 50, Australia). The percentage free radical scavenging activity (RSA) of crude plant extracts was calculated according to the formula:

\[
RSA(\%) = 100 \times \left(1 - \frac{A_c}{A_B}\right)
\]

where, \(A_c\) is the absorbance of plant extracts and/or standard antioxidants prepared without the DPPH solution subtracted from the absorbance value of corresponding samples prepared with DPPH solution and; \(A_B\) is the absorbance value of the negative control.

The \(\beta\)-carotene–linoleic bioassay explores the antioxidant capacity of a compound by testing its ability to donate electrons with the resulting peroxyl radical. The capacity of crude methanolic orchid extracts to prevent or reduce the coupled oxidation of \(\beta\)-carotene and linoleic acid in an emulsified aqueous system (Pajero et al., 2002) was determined using the protocol outlined by Aamarowicz et al. (2004) with modifications. The entire assay was carried out under green light conditions as \(\beta\)-carotene is light sensitive. \(\beta\)-carotene (10 mg) was dissolved in 10 ml of chloroform to give a final concentration of 0.1 g/ml in a brown Schott bottle. Excess chloroform was evaporated under vacuum, leaving a thin film of \(\beta\)-carotene, to which 200 µl of linoleic acid and 2 ml of polyoxyethylene sorbitan monolaurate (Tween 20) were added. The total volume was made up to 500 ml with aerated distilled water with the final concentration of \(\beta\)-carotene being 20 µg/ml. The mixture was saturated with oxygen by vigorous agitation to form an orange emulsion. The \(\beta\)-carotene emulsion (4.8 ml) was dispensed into glass test tubes, followed by 200 µl of either plant samples or positive control BHT (6.25 mg/ml). The effective concentration of samples and positive control was 250 µg/ml in the reaction mixtures. The negative control consisted of 50% methanol. Each sample was prepared and tested in triplicate. The absorbance of each reaction mixture was measured at 470 nm using a spectrophotometer (Varian Cary 50, Australia) after which, reaction vessels were incubated for 2 h at 50 °C in the dark. The absorbance of each reaction mixture was measured every 30 min during the incubation period at 470 nm. Tween 20 was used to blank the spectrophotometer.

Antioxidant activities were expressed as average antioxidant activity (%ANT) and oxidation rate ratio (ORR). The rate of \(\beta\)-carotene bleaching was calculated according to first order kinetics using the formula:

\[
\text{Rate of } \beta\text{-carotene bleaching} = \left[\frac{A_{t=0}}{A_{t=t}}\right] \times 1/t
\]

where, \(A_{t=0}\) is the absorbance of the emulsion at 0 min; and \(A_{t=t}\) is the absorbance at time \(t\) (30, 60, 90 min). The average rate of \(\beta\)-carotene bleaching was calculated based on rates at 30, 60 and 90 min. The calculated average rates were used to determine the %ANT of the respective extracts, and expressed as percent inhibition of the rate of \(\beta\)-carotene bleaching in relation to the negative control using the formula:

\[
\% \text{ ANT} = \frac{R_{\text{control}} - R_{\text{sample}}}{R_{\text{control}}} \times 100\%
\]

where, \(R_{\text{control}}\) and \(R_{\text{sample}}\), represent the respective average \(\beta\)-carotene bleaching rates for the standard antioxidant and plant extracts, respectively. Antioxidant activity was further expressed as the ORR based on the formula:

\[
\text{ORR} = \frac{R_{\text{sample}}}{R_{\text{control}}}
\]

The ferric reducing power assay (FRAP) outlined by Lim et al. (2009) was used to determine the ability of crude plant extracts to reduce the Fe\(^{3+}\)/ferriyricyanide complex to the ferrous (Fe\(^{2+}\)) form. Using a 96 well microtiter plate, 30 µl of methanolic plant extract (6.25 mg/ml), ascorbic acid (0.8 mM) or BHT (0.5 mM) was serially diluted in 30 µl of Millipore water. Thereafter, 40 µl of 0.2 M phosphate buffer (pH 7.2) and 40 µl of potassium ferricyanide (1% w/v in potassium phosphate buffer) were added consecutively to each well. Test solutions were incubated for 20 min at 50 °C, followed by the addition of trichloroacetic acid (40 µl, 10% w/v), distilled water (150 µl) and ferric chloride (30 µl, 0.1% w/v in potassium phosphate buffer). Samples were incubated for a further 30 min at room temperature in the dark. The absorbance was read at 630 nm using a spectrophotometer (Varian Cary 50, Australia); where an increase in absorbance indicated a greater reducing power. Samples were prepared and tested in triplicate.

2.5. Ames salmonella/microsome mutagenicity assay (Ames test)

The in vitro mutagenicity assay was carried out using three histidine-deficient (his-) Salmonella typhimurium bacterial strains TA98, TA100 and TA102 (Maron and Ames, 1983). Overnight cultures of each strain, representing different mutations, were incubated in Oxoid nutrient broth No. 2 on an orbital shaker over a 16 h period at 37 °C to produce a density of 2×10⁹ colony forming units (CFU/ml). A dilution series of the crude plant extracts (50, 500 and 5000 µg/ml) was prepared by re-suspending extracts in 10% DMSO. A positive control, 4-nitroquinoline-1-oxide (4NQO), was prepared from a stock solution of 2 mg/ml to a concentration of 2 µg/ml.

Using aseptic techniques throughout the assay, 100 µl of each test solution (sample and control solutions) were dispensed into culture tubes, followed by 500 µl of sodium phosphate buffer (0.1 M pH 7.4). Following a 3 min incubation period, 100 µl of overnight bacterial cultures (2 × 10⁸ cells/ml (CFU/ml) was added to each tube. Thereafter, 10 ml of 0.5 mM histidine/biotin solution was added to the liquid top agar to produce an enriched agar; of which 2 ml was added to each test solution. The test solution was then poured over the surface of labelled minimal glucose agar plates and incubated (inverted in the dark) at 37 °C for 48 h. For the experiment with S9 metabolic activation, a freshly prepared S9 mixture containing 5% (v/v) S9 fraction sourced from Sprague–Dawley male rats (Sigma-Aldrich, Co., St Louis) in mixed enzymic cofactors with NADP was kept on ice and was used in place of the phosphate buffer (500 µl) mentioned in the above experiment. The positive control 2-aminoanthracene (2AA) (2 µg/ml) was used. At the end of the incubation period, the number of revertant colonies was counted using an electronic counter.

A crude extract was considered to be mutagenic if the number or revertant colonies were twice the number found in the negative controls and showed a dose-dependent curve. The mutagenic index (MI) was also determined; where a MI ≥ 2 and a dose-dependent response were considered mutagenic (Varella et al., 2004).

The EC₅₀ for each sample was determined from a logarithmic non-linear regression curve using GraphPad Prism software version 4.03 (GraphPad Software, Inc., San Diego, USA). A one-way analysis of variance (ANOVA) and Duncan Multiple Range Test of mean ± standard error of mean (SEM) was used to determine differences and significant differences among means; using SPSS software version 10 (SPSS Inc., Chicago, USA). P = 0.05 indicated significantly different samples.
3. Results

3.1. Cyclooxygenase enzyme inhibition

Anti-inflammatory activity above 70% was considered significant (Taylor and Van Staden, 2001). Out of a total of 53 evaluated extracts, 21 and 13 extracts exhibited significant anti-inflammatory activity in the COX-1 and COX-2 assays respectively (Table 1). All aqueous extracts, except that of *A. africana* roots and *B. scaberulum* pseudobulbs, showed poor or no COX-1 and COX-2 inhibition. The DCM tuber extract of *E. hereroensis* was the only extract to significantly inhibit both COX enzymes, 100.02 ± 0.11% and 87.97 ± 8.38% respectively. All *B. scaberulum* organic root extracts exhibited COX-2 selective inhibitory activity.

The EC₅₀ of crude orchid extracts against COX-1 and COX-2 is presented in Table 2. Based on the anti-inflammatory activity presented in Table 1, the EC₅₀ of 14 crude extracts were determined in the COX-1 assay; and six crude extracts were determined in the COX-2 assay. Overall, the DCM root extract of *A. africana* was found to be the most potent extract (0.25 ± 0.10 mg/ml). This extract was the most potent extract in the COX-1 assay, while the 80% EtOH root extract of *B. scaberulum* was the most potent in the COX-2 assay (0.44 ± 0.32 mg/ml).

3.2. Anti-cholinesterase activity

The AChE inhibitory activity of different crude extracts of seven orchid species is presented in Table 3. Generally it was the root extracts which exhibited greater AChE inhibitory activity. The lowest EC₅₀ value Table 1

| Species                | Plant part | Extraction solvent | Percent inhibition (%) |
|------------------------|------------|--------------------|------------------------|
| Ansellia africana      | Leaves     | PE                 | ND                     |
|                        |            | DCM                | 66.03 ± 13.64          |
|                        |            | Ethanol            | 57.48 ± 20.80          |
|                        |            | Water              | 104.11 ± 1.62          |
|                        | Stems      | PE                 | 100.00 ± 0.00          |
|                        |            | DCM                | 106.11 ± 1.29          |
|                        |            | Ethanol            | 78.52 ± 0.97           |
|                        |            | Water              | 19.51 ± 4.73           |
|                        | Roots      | PE                 | 16.08 ± 11.03          |
|                        |            | DCM                | 99.36 ± 1.35           |
|                        |            | Ethanol            | 17.21 ± 1.50           |
|                        |            | Water              | 57.48 ± 20.80          |
| Bulbophyllum scaberulum| Leaves     | Water              | ND                     |
|                        |            | DCM                | 106.11 ± 1.29          |
|                        |            | Ethanol            | 78.52 ± 0.97           |
|                        |            | Water              | 19.51 ± 4.73           |
|                        | Roots      | DCM                | 100.06 ± 0.01          |
|                        |            | Ethanol            | 93.31 ± 2.33           |
|                        |            | Water              | 57.48 ± 20.80          |
| Cyrtorchis arcuata     | Leaves     | Ethanol            | ND                     |
|                        |            | Water              | 89.19 ± 0.18           |
|                        | Roots      | Ethanol            | 76.75 ± 11.02          |
|                        |            | Water              | ND                     |
|                        | DCM        | 106.11 ± 1.29      |
|                        | Ethanol    | 78.52 ± 0.97       |
|                        | Water      | 19.51 ± 4.73       |
|                        | Ethanol    | 100.06 ± 0.01      |
|                        | Water      | 93.31 ± 2.33       |
| Eulophia hereroensis   | Tubers     | PE                 | ND                     |
|                        |            | DCM                | 100.02 ± 0.11          |
|                        |            | Ethanol            | 87.97 ± 8.38           |
|                        |            | Water              | 58.09 ± 3.25           |
|                        | Roots      | Ethanol            | ND                     |
|                        |            | Water              | 76.75 ± 11.02          |
|                        | DCM        | 100.02 ± 0.11      |
|                        | Ethanol    | 87.97 ± 8.38       |
|                        | Water      | 58.09 ± 3.25       |
| Eulophia petersii      | Leaves     | PE                 | ND                     |
|                        |            | DCM                | 109.88 ± 1.48          |
|                        |            | Ethanol            | 27.30 ± 9.78           |
|                        |            | Water              | 22.16 ± 13.85          |
|                        | Roots      | Ethanol            | ND                     |
|                        |            | Water              | 74.27 ± 10.14          |
|                        | DCM        | 109.88 ± 1.48      |
|                        | Ethanol    | 27.30 ± 9.78       |
|                        | Water      | 22.16 ± 13.85      |
|                        | Ethanol    | 109.88 ± 1.48      |
|                        | Water      | 27.30 ± 9.78       |
|                        | Ethanol    | 22.16 ± 13.85      |
|                        | Water      | 74.27 ± 10.14      |
|                        | Ethanol    | 30.16 ± 2.67       |
|                        | Water      | 10.16 ± 12.50      |
|                        | Ethanol    | 24.91 ± 0.86       |
|                        | Water      | 15.05 ± 8.06       |
|                        | Ethanol    | 30.16 ± 2.67       |
|                        | Water      | 24.91 ± 0.86       |
|                        | Ethanol    | 15.05 ± 8.06       |
|                        | Water      | 24.91 ± 0.86       |
|                        | Ethanol    | 30.16 ± 2.67       |
|                        | Water      | 10.16 ± 12.50      |
| Polystachya pubescens  | Pseudobulbs| DCM                | ND                     |
|                        |            | Ethanol            | 24.91 ± 0.86           |
|                        |            | Water              | 15.05 ± 8.06           |
|                        | Roots      | Ethanol            | ND                     |
|                        |            | Water              | 24.91 ± 0.86           |
|                        | Ethanol    | 15.05 ± 8.06       |
|                        | Water      | 24.91 ± 0.86       |
|                        | Ethanol    | 15.05 ± 8.06       |
|                        | Water      | 24.91 ± 0.86       |
| Tridactyle tridentata  | Leaves     | Water              | 45.67 ± 2.45           |
|                        | Roots      | DCM                | 45.67 ± 2.45           |
|                        | Ethanol    | 45.67 ± 2.45       |
|                        | Water      | 45.67 ± 2.45       |
|                        | Ethanol    | 45.67 ± 2.45       |
|                        | Water      | 45.67 ± 2.45       |

Values are the mean (±) standard deviation (n = 3). ND—not determined. Anti-inflammatory activity below 20% was considered insignificant, 20–40% low, 40–70% moderate and above 70% was considered significant (Taylor and Van Staden, 2001); values in bold indicate those extracts where an EC₅₀ was determined.
was observed in B. scaberulum DCM root extract (0.02 ± 0.00 mg/ml). The most active extract of A. africana was the EtOH root extract. For C. arcuata the aqueous root extract proved to be the most potent. The most effective extracts of E. hereroensis and E. petersii were the EtOH root and DCM stem extracts respectively. The DCM root extract was the most potent extract for P. pubescens, while the most active extract of T. tridentata was the EtOH root extract.

### 3.3. Antioxidant activities

Percentage free radical scavenging activity of crude orchid extracts were measured in the DPPH radical scavenging assay. While the principle of the assay does not stipulate the meaning of negative percentage radical scavenging activity the use of a wide range of concentrations ensured the determination of an EC50 value. There was a dose-dependent change in radical scavenging activities of crude extracts from which EC50 Values were determined. In terms of EC50, the methanolic root extracts of all species, except that of E. petersii, had consistently more effective radical scavenging activity than that of other plant parts within each species. The methanolic pseudobulb extract of E. petersii was the most potent extract (1.32 ± 0.86 mg/ml).

The antioxidant activity of crude orchid extracts as measured in the β-carotene-linoleic acid assay is presented in Table 4. Beta carotene bleaching occurs because of oxidation of linoleic acid; this coupled oxidation was observed by a decrease in absorbance over time. Based on ORR, the leaf extract of T. tridentata and the root extracts of C. arcuata and E. hereroensis exhibited the best antioxidant effects (0.02, 0.023 and -0.15 respectively). Similarly, the %ANT of these samples was greater than that of BHT (95.88 ± 6.90%) and all other samples. Eight out of a total of 18 samples exhibited a greater capacity to prevent β-carotene oxidation in the assay when compared to BHT.

### 3.4. Mutagenic effects

Table 5 presents the average number of induced revertant colonies, the standard deviation and the MI at different concentrations (5, 0.5, 0.05 mg/ml) of crude orchid extracts using three S. typhimurium strains (TA98, TA100 and TA102). A crude extract was considered to be mutagenic if the number or revertant colonies were twice the number found in the negative controls and showed a dose-dependent response. Mutagenic extracts were observed for the TA98 strain only. All concentrations of A. africana DCM leaf and stem extracts tested, the DCM root extract (5, 0.5 mg/ml) and EtOH leaf, stem and root extracts at 5 mg/ml exhibited mutagenic effects. The EtOH root extracts (5, 0.05 mg/ml) of B. scaberulum exhibited mutagenic indices comparable to that of 4NQO (17.00 and 13.00, respectively). Eulophia petersii PE pseudobulb extract demonstrated mutagenic potential at the highest concentration tested (5 mg/ml). The EtOH root extracts of T. tridentata showed mutagenic effects at 0.5 and 0.05 mg/ml.
Table 4
Percent inhibition of β-carotene bleaching by crude methanol extracts of seven indigenous South African orchids.

| Species          | Plant part | ORR   | % ANT      |
|------------------|------------|-------|------------|
| Ansellia africana| Leaves     | 0.42 ± 0.31a | 87.87 ± 2.74bc |
|                  | Stems      | 0.31 ± 0.01a  | 90.61 ± 5.25bc |
|                  | Roots      | ND     | ND         |
| Bulbophyllum scaberulum | Leaves     | 0.20 ± 0.61a  | 100.00 ± 55.61e |
|                  | Pseudobulbs| 0.1 ± 0.001a  | 100.00 ± 5.23e  |
|                  | Roots      | 1.22 ± 1.37a  | 100.00 ± 1.32e  |
| Cyrtorchis arcuata| Leaves     | 0.35 ± 0.49a  | 29.45 ± 4.22e  |
|                  | Roots      | 0.023 ± 0.04a | 100.00 ± 10.48c |
| Eulophia hereroensis| Tubers    | 0.63 ± 0.44a  | 63.96 ± 22.98g  |
|                  | Roots      | 0.001 ± 0.18a | 100.00 ± 8.41e  |
| Eulophia petterisi | Leaves    | 0.31 ± 0.18a  | 84.96 ± 3.10e  |
|                  | Stems      | 0.28 ± 0.02a  | 92.18 ± 2.10e  |
|                  | Pseudobulbs| 0.22 ± 0.07a  | 98.62 ± 0.50med |
|                | Roots      | 0.19 ± 0.10a  | 92.42 ± 0.001b |
| Polystachya pubescens | Pseudobulbs| 0.28 ± 0.14a  | 92.75 ± 2.78e  |
|                  | Roots      | 0.23 ± 0.09a  | 90.12 ± 2.89e  |
| Tridactyle tridentata | Leaves    | 0.02 ± 0.18a  | 100.00 ± 25.74e |
|                  | Roots      | 0.10 ± 0.01a  | 96.66 ± 1.000md |

Values are the mean (±) standard error (n = 2); EC50 values in bold indicate those extracts with most significant antioxidant activity. ORR = Average β-carotene bleaching rates as compared to the negative control and %ANT = average antioxidant activity; and %ANT for BHT (95.88 ± 6.90%). Different letters in each column represent significance differences between means, P = 0.05.

Table 6 presents the average number of induced revertant colonies, the standard deviation and the MI at different concentrations (5, 0.5, 0.05 mg/ml) of crude orchid extracts using S. typhimurium strain TA98 with metabolic activation. A crude extract was considered to be mutagenic if the number of revertant colonies were twice the number found in the negative controls and showed a dose-dependent response. None of the extracts tested demonstrated mutagenic effects.

4. Discussion

The effectiveness of anti-inflammatory and antioxidant drugs in treating inflammatory and neurodegenerative disorders has been widely documented (Houghton et al., 2007; Howes and Houghton, 2003). The current study demonstrated the anti-inflammatory, antioxidant and AChE inhibitory activities of various crude orchid extracts. Approximately five species of South African orchids are used to treat inflammatory conditions. Polystachya ottoniana is used to soothe pain experienced in teething babies and to treat diarrhoea. A. africana is administered as an antimicrobial while Eulophia species such as Eulophia cucullata and Eulophia ovalis, are used primarily to relieve pain. The current investigation included research of C. arcuata (treatment of diabetes and skin infections) and T. tridentata (treatment of psychological disorders such as madness). Two orchid species, B. scaberulum and E. hereroensis, not mentioned in literature previously used in South African traditional medicine were also included as they were being traded in the abovementioned herbal markets.

Orchid extracts that displayed significant effects in anti-inflammatory, antioxidant and AChE inhibitory assays may be potent natural plant product targets in the treatment of inflammatory and neurodegenerative disorders. Extracts include: A. africana EtOH root, B. scaberulum DCM root, C. arcuata methanolic root, E. hereroensis DCM tuber, E. petterisi DCM stem and T. tridentata DCM root extracts. The EtOH root extract of B. scaberulum exhibited the most potent selective inhibitory effect on COX-2 (Table 2). While the DCM tuber extract of E. hereroensis, was the only extract to significantly inhibit both COX enzymes (Table 2). Preliminary tests suggest significantly higher levels of gallotannin content in A. africana, and E. hereroensis methanol root extracts. This may account for the significant anti-inflammatory activity. Similarly, the presence of condensed tannins in E. hereroensis root and B. scaberulum stem/root extracts may explain the observed anti-inflammatory effects.

The potent anti-inflammatory and antioxidant effect of E. hereroensis and E. petterisi supports the use of species from this genus for inflammatory-related symptoms in South African traditional medicine. The overall %ANT of P. pubescens pseudobulb and root extracts was greater than 90%, which might validate the use of species from this genus as substitutes to P. ottoniana, to treat certain inflammatory disorders. Flavonoids in the pseudobulbs and roots of P. pubescens may have contributed to the antioxidant effects. During the survey of leaf flavonoid content in Orchidaceae, Williams (1979) isolated xanthones, mangiferin and isomangiferin from five species of Polystachya and Maxillaria. The author also observed that there was no pattern of flavonoid distribution within the family Orchidaceae, and geographical location played a significant role in the presence of flavonoid compounds (Williams, 1979). All four species that did register some flavonoid content in preliminary studies, shared similar distribution ranges; and are all epiphytic species. Plant compounds such as flavonoids, naphthoquinones, alkaloids and phenolic phenyl-propane derivatives represent the usual compounds found in certain natural products that are responsible for COX inhibition (Bohlin, 1995). The presence of flavonoids in B. scaberulum and T. tridentata may explain the potent activity observed in the anti-inflammatory and AChE inhibitory assays. The medicinal value of flavonoids includes anti-inflammatory, antifungal, antioxidant activities and wound healing. The wound healing efficacy of Oncidium flexuosum, an epiphytic orchid used in Brazilian traditional medicine for inflammation and wounds, was attributed to the presence of flavonoids and tannins (De Gaspi et al., 2011). Imbricatin, a stilbenoid isolated previously from Bulbophyllum and other orchid genera, was one of three isolated stilbenoids recommended for use as skin photoprotectants based on antioxidant, anti-inflammatory and immunomodulatory effects (Simmler et al., 2010). The Bulbophyllum and Eulophia genera are not only traded in South African herbal markets for medicinal uses, they also feature in a list of 21 genera listed in Ayurvedic literature as being used for medicines (Singh et al., 2007). The medicinal usefulness of the Bulbophyllum and Eulophia genera is supported by the pharmacological and phytochemical assessment of the genus Vanda which also features on this list of common medicinal plant sources in Indian traditional medicine (Singh et al., 2007). Simmler et al. (2010) evaluated the skin photoprotective effect of Vanda coerulea stilbenoids based on the widespread use of orchid extracts in the cosmetic industry. Ultraviolet-induced damage often results in oxidative stress and associated inflammatory processes. Ultraviolet B (UVB) radiation is known to increase PGE2 production (due to the upregulation of COX-2 expression) and ROS; resulting in skin inflammation. Simmler et al. (2010) mention natural photoprotective polyphenol compounds such as proanthocyanidins and resveratrol with proven antioxidant and related anti-inflammatory activities. Stem methanol solutions of isolated stilbenoids from V. coerulea performed better than “hydro-alcoholic” solutions in the in vitro DPPH/O radical scavenging and Parameter™ PGE-2 enzyme immunological assays. Further chemical analysis of South African Bulbophyllum and Eulophia species may reveal related chemical compounds with comparable results.

When testing methanolic crude extracts in two different SET reaction-based assays, the E. petterisi pseudobulb extract was the most potent radical scavenging extract (Fig. 1). Alternatively, when using the β-carotene-linoleic acid assay, a HAT reaction-based assay, the ORR revealed the leaf extract of T. tridentata and the root extracts of C. arcuata and E. hereroensis to have the best antioxidant effects (Table 4). Similarly, the overall %ANT of these three samples to prevent the coupled oxidation of β-carotene and linoleic acid were greater than that of BHT and all other samples. The total phenolic content of E. hereroensis pseudobulb extracts may contribute to the antioxidant activity; while the presence of flavonoids in the leaf and root extracts of T. tridentata and C. arcuata, respectively, may play a role in the
The antioxidant activity observed in the HAT reaction-based assay. An alternative mechanism may have been affected due to the presence of the flavonoids in these extracts.

The medicinal uses of *C. arcuata* (treatment of diabetes and skin infections) and *T. tridentata* (treatment of psychological disorders such as madness) may be validated based on their significant anti-inflammatory and antioxidant effects (Tables 1, 2, 5) (Bulpitt, 2005). The anti-inflammatory and antioxidant activities demonstrated in this investigation may suggest cellular and neuroprotective effects. Different classes of well-established anti-inflammatory pathways have been shown to exhibit varied effects in patients with neuroinflammatory disorders such as Alzheimer’s. With prolonged use, other NSAIDs have demonstrated a positive response (DeKosky, 2003). Such varied responses to anti-inflammatory drugs, is indicative of alternative neuroprotective pathways other than COX-inhibition (Stuchbury and Münch, 2005). This observation, and the potential of COX-inhibitors in treatment of Alzheimer’s, is further supported by the report of Montine et al. (1999) where COX activity in persons susceptible to Alzheimer’s was comparable to that of control persons; while concentration levels of PGE2 in cerebrospinal fluid of susceptible patients had increased five times. *Cyrtochis arcuata* and *T. tridentata* also share a similar distribution pattern, which may result in the accumulation of similar bioactive compounds and account for the comparable antioxidant effects. The use of the same vernacular name for *C. arcuata*, various *Eulophia* species and *Tridactyle* species, may be explained by their comparable positive anti-inflammatory and antioxidant effects in this investigation.

Based on the assumption that users of traditional medicine would consume an aqueous concoction of plant material, aqueous extracts were expected to exhibit significant biological activity. Instead, all aqueous extracts, except that of *A. africana* roots and *B. scaberulum* pseudobulbs, showed poor or no COX-1 and COX-2 inhibition (Table 1). Higher levels of activity were achieved with the organic extracts (PE, DCM and EtOH) (Table 1). Root extracts of *B. scaberulum* exhibited the most potent selective inhibitory effect on COX-2 (Table 2) and interestingly, the DCM root extract was also the most effective crude orchid extract to inhibit AChE; performing better than the commercial product galanthamine (Table 3). Natural plant constituents such as polyphenols, saponins, certain pigments and even fatty acids are often extracted when using non-polar solvents (Lower, 1985). The EtOH extract of *Gastrodia elata*, traditionally used to treat inflammatory disorders such as rheumatism, demonstrated potent anti-angiogenic effects in the CAM assay; while the n-butanol fraction showed dose-dependent activity. In vivo vascular permeability, an early sign of inflammatory response, was significantly inhibited; while strong analgesic and anti-inflammatory activity was observed. Ahn et al. (2007) postulated that such activity was a result of inhibited NO production and COX-2 expression. Compounds such as 4-hydroxybenzyl alcohol and 4-hydroxybenzaldehyde, isolates of *G. elata*, were thought to be responsible for the anti-inflammatory effects (Ahn et al., 2007).

Researchers, looking for compounds with more extensive biological activities, produced a new class of active and selective AChE inhibitors by introducing amino acid moieties into the backbone of 4-hydroxybenzaldehyde. The resultant compounds exhibited more potent AChE inhibitory activity. Two compounds 4b (IC50 0.19 μM) and 4i (IC50 0.28 μM) performed better than galanthamine (IC50 0.55 μM); and 4i was selective for AChE (Wen et al., 2007).

Alternatively, traditional healers may be using hot water extracts (as opposed to our cold water extracts); a procedure that could possibly extract the less water-soluble compounds when using water as the extractant. Primary metabolites such as carbohydrates are commonly extracted with water (Zha et al., 2007). *Cyrtopodium cardiochilum* (CC) hot water pseudobulb extract produced the CC polysaccharide. It was found to significantly increase the phagocytic index when compared to the standard (Zymosan) and exhibited a similar significant anti-inflammatory effect when compared to Indomethacin (10 mg/kg body weight). Where an increase in vascular permeability is one of the...
early signs of an inflammatory response; the CCP glucocmanann suppressed this response by approximately 20% of the control (Barreto and Parente, 2006).

Mainly due to safety concerns in pharmaceutical and food industries, mutagenicity assessment programmes have increased dramatically. There are two major concerns regarding mutagens, these are: their ability either to induce cancer or to affect the germ line and future generations. In industry the Salmonella mutagenicity (Ames) test, which detects gene mutations (Verschaeve et al., 2004), is used extensively as a preliminary indicator to identify possible carcinogens and/or the mutagenic potential of crude plant extracts and clinical drugs. However, not all cancer causing substances show positive results in the Ames test. A positive result indicates the ability of a substance to produce a change in DNA structure; that is, gene point mutations or the gain or loss of whole chromosomes. However, further analysis is required to determine the carcinogenic potential of the substance. Elgorashi et al. (2003) reported on the possible mutagenic effects of 51 South African medicinal plant species. Reid et al. (2006) evaluated 42 other South African species. There has been extensive research into the mutagenic and anti-mutagenic potential of medicinal plants used in the South African traditional medical system (Elgorashi et al., 2002).

Medicinal orchids were selected on the basis of their ethnobotanical use and availability. A plant extract was considered mutagenic when a MI of ≥2 for any concentration of that extract was observed; together with a dose-dependent response (Vargas et al., 1993). An increase in number of His+ revertant colonies over the negative control indicated genotoxic effects. The Ames assay revealed that mutagenic extracts were observed for the TA98 strain only. Without metabolic activation, certain organic extracts of A. africana leaf, stem and root extract at various concentrations tested (0.5 and 0.05 mg/ml) exhibited genotoxic effects; while the EtOH root extracts (5 and 0.5 mg/ml) of B. scherulatum exhibited mutagenic indices comparable to that of 4NQO. Bioactive E. petersii PE pseudobulb extract demonstrated mutagenic potential at the highest concentration tested (5 mg/ml). The EtOH root extracts of

| Table 5 | Mutagenicity of crude orchid extracts as determined in the Salmonella/microsome assay (without metabolic activation) expressed as average revertants per plate and mutagenicity index. |
|------------------|------------------|------------------|------------------|------------------|
| Species          | Plant part       | Extraction solvent | Concentration (µg/ml) | Salmonella typhimurium strains |
|                  |                  |                  | 500              | 5000                  |
|                  |                  |                  | TA98 Revertants per plate | Mutagenicity index |
|                  |                  |                  | TA100 Revertants per plate | Mutagenicity index |
|                  |                  |                  | TA102 Revertants per plate | Mutagenicity index |
|                  |                  |                  | 500              | 5000                  |
| Ansellia africana | Leaves           | DCM              | 47.34 ± 12.3     | 3.95                  |
|                  |                  |                  | 36.67 ± 7.1      | 3.06                  |
|                  |                  |                  | 32.67 ± 1.9      | 2.72                  |
|                  |                  |                  | 25.34 ± 10.4     | 2.11                  |
|                  |                  |                  | 20.17 ± 4.9      | 1.68                  |
|                  |                  |                  | 12.67 ± 1.2      | 1.06                  |
|                  |                  |                  | 41.00 ± 6.6      | 3.42                  |
|                  |                  |                  | 26.00 ± 1.4      | 2.17                  |
|                  |                  |                  | 34.00 ± 6.1      | 2.83                  |
|                  |                  |                  | 27.00 ± 9.9      | 2.25                  |
|                  |                  |                  | 22.84 ± 6.4      | 1.90                  |
|                  |                  |                  | 18.17 ± 1.2      | 1.51                  |
|                  |                  |                  | 36.50 ± 12.0     | 3.04                  |
|                  |                  |                  | 31.50 ± 9.19     | 2.63                  |
|                  |                  |                  | 22.50 ± 3.54     | 1.88                  |
|                  |                  |                  | 31.50 ± 9.19     | 2.63                  |
|                  |                  |                  | 11.50 ± 4.95     | 0.96                  |
| Bulbophyllum sp. | Root              | DCM              | 20.50 ± 6.36     | 1.71                  |
|                  |                  |                  | 18.50 ± 9.19     | 1.54                  |
|                  |                  |                  | 18.00 ± 9.90     | 1.50                  |
|                  |                  |                  | 204.00 ± 31.1    | 17.00                 |
|                  |                  |                  | 156.00 ± 28.28   | 13.00                 |
|                  |                  |                  | 115.0 ± 4.95     | 0.96                  |
| Cyrtorchis arcuata| Leaf              | PE               | 18.00 ± 4.24     | 1.50                  |
|                  |                  |                  | 15.50 ± 7.78     | 1.29                  |
|                  |                  |                  | 13.50 ± 0.71     | 1.13                  |
|                  |                  |                  | 16.00 ± 5.66     | 1.33                  |
|                  |                  |                  | 11.50 ± 4.95     | 0.96                  |
|                  |                  |                  | 9.00 ± 0.90      | 0.75                  |
|                  |                  |                  | 7.50 ± 0.71      | 0.63                  |
|                  |                  |                  | 12.50 ± 2.12     | 1.04                  |
|                  |                  |                  | 7.50 ± 6.36      | 0.63                  |
|                  |                  |                  | 8.50 ± 0.71      | 0.71                  |
|                  |                  |                  | 13.00 ± 1.41     | 1.08                  |
|                  |                  |                  | 9.00 ± 2.83      | 0.75                  |
|                  |                  |                  | 11.50 ± 2.12     | 0.96                  |
|                  |                  |                  | 13.50 ± 4.95     | 1.13                  |
|                  |                  |                  | 15.00 ± 2.82     | 1.25                  |
|                  |                  |                  | 14.50 ± 2.12     | 1.21                  |
|                  |                  |                  | 15.50 ± 4.95     | 1.29                  |
|                  |                  |                  | 11.50 ± 0.71     | 0.96                  |
|                  |                  |                  | 15.00 ± 2.82     | 1.25                  |
|                  |                  |                  | 18.00 ± 0.00     | 1.50                  |
|                  |                  |                  | 17.50 ± 2.12     | 1.46                  |
|                  |                  |                  | 11.00 ± 1.41     | 0.92                  |
|                  |                  |                  | 14.00 ± 5.66     | 1.17                  |

(continued on next page)
T. tridentata showed mutagenic effects at 5 and 0.5 mg/ml. With metabolic activation using TA98, no mutagenic effects were observed for all crude orchid extracts tested.

The primary aim of mutagenicity testing of bioactive natural plant extracts is to determine their safety. Based on available literature, there are limited reports of mutagenicity and safety testing of orchid extracts and their isolated products. Of the seven orchid species considered in this investigation, organic leaf and root extracts of C. arcuata produced no genotoxic effects (Table 5). The extracts may contain flavonoids. Food derived flavonols such as quercetin, kaempferol and myricetin have been reported to affect the cell cycle and growth of cancer cells, thereby reducing the risk of cancer (Verma et al., 1988; Yoshida et al., 1990). The non-genotoxic effects of C. arcuata coupled with the presence of flavonoids necessitates further investigation. Zhang et al. (2005) also established that dimersed phenantherenes are required to inhibit cancer cell growth. Research on compounds such as moscatnlin; with known anti-inflammatory, cytotoxic, anti-platelet aggregation and anti-proliferative properties; and erianin, provide insight into the mechanisms involved in cancer progression. Similar, orchid extracts that demonstrate significant biological activities should be researched further for other biological activities. The herbicidal potential of gymnuspin, a phenantherine derivative from Maxillaria densa,
was weak as it displayed cytotoxic effects in vitro against four mammalian cell lines H4TG (IC$_{50}$ 13.0 ± 0.9 μM), MDCK (IC$_{50}$ 11.0 ± 0.5 μM), NH3T3 (IC$_{50}$ 12.0 ± 1.0 μM) and KA31T (IC$_{50}$ 21.0 ± 0.5 μM) (Valencia-Islas et al., 2002). Collectively, such information can benefit further research efforts dealing with bioherbicides and their safety.

Conversely, extensive research has been conducted on the antitumor, antimutagenic and anti-angiogenesis effects of orchid derived extracts and compounds. While some cytotoxic compounds are considered a biohazard, some compounds are often referred to as anticancer compounds (Kovács et al., 2008). Antitumour activities have been detected in the Bulbophyllum genus (Yao et al., 2005), with Bulbophyllum odoratissimum, in particular, containing cytotoxic phenolics (Chen et al., 2008). Antitumour activities have been detected further research efforts dealing with bioherbicides and their safety. Generally the results obtained in this study validate the orchid-derived medicines are on the human body and more especially used for cultural practices are administered as emetics (Chinsamy et al., 2011).

The toxicology and mutagenicity of medicinal plants need equal consideration in medicinal plant research. Most of the orchid species used for cultural practices are administered as emetics (Chinsamy et al., 2011). It would be important to know what the effects of these orchid-derived medicines are on the human body and more especially their safety. Generally the results obtained in this study validate the use of certain orchid species in South African traditional medicine for pain-related ailments. Other pharmacological conditions linked to inflammation were also investigated to establish other potential activities of the medicinal plant extracts. A more comprehensive assessment of the chemistry of South African orchids would allow one to more confidently assert a relationship between chemical profiles, interaction between different classes of compounds, biological activity and influence of geographical location. The results obtained in this study provide scientific information that could aid in the isolation of potential bioactive compounds with fewer side-effects.

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