Investigation of *in vitro* and *in vivo* anti-asthmatic properties
of *Siphonochilus aethiopicus*

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Abstract

Aim of the study: Asthma is a chronic inflammatory disease of the lungs, characterized by increased sensitivity to bronchoconstriction associated with infiltration of immune cells, mucus hypersecretion and structural remodelling of the airways. In South Africa, the indigenous plant *Siphonochilus aethiopicus*, is used by traditional health practitioners to treat colds, wheezing of the chest, coughs, influenza, sinus problems and mild asthma. In this study we aimed to investigate the potential anti-inflammatory and anti-allergic properties of *S. aethiopicus* in vitro and its efficacy in a mouse model of allergic asthma.

Materials and methods: The dried and powdered *S. aethiopicus* plant material was extracted separately with organic solvents (diethyl ether, ethanol) and water. Dried extracts as well as a purified furanoterpenoid compound present in the extracts were screened in vitro in a glucocorticoid and histamine H₁ receptor binding assay and a phosphodiesterase IV enzyme inhibition assay. Extracts were also evaluated for efficacy against ovalbumin (OVA)-induced allergic airway disease in mice.

Results: Biological assaying of extracts of the plant and the isolated furanoterpenoid showed significant in vitro inhibition of glucocorticoid and histamine H₁ receptor binding and phosphodiesterase IV activity, supporting a possible anti-inflammatory, anti-allergic and bronchodilatory effects. Administration of *S. aethiopicus* extracts to OVA-sensitized and challenged mice significantly reduced lung inflammation and the percentage of eosinophils in bronchoalveolar lavage fluid but did not influence airway hyper reactivity.
Conclusion: This study provides evidence that *S. aethiopicus* has anti-inflammatory and anti-allergic properties *in vitro* and *in vivo*. These findings may support anecdotal accounts of its effectiveness against asthma, sinusitis, colds and flu.
1. Introduction

*Siphonichilus aethiopicus* is a member of the family Zingiberaceae whose natural distribution includes tropical and southern Africa (Monocot Checklist), including South Africa, Zimbabwe, Malawi and Zambia (Holzapfel et al., 2002). *S. aethiopicus* is commonly known as *African ginger* and is widely used in traditional medicine throughout South Africa where in Zulu it is known as ‘*isiphephetho*’ or ‘*indungulo*’ (Van Wyk et al., 2009). It is a deciduous plant with large leaves developing annually from a small cone-shaped rhizome. The rhizomes and freshly roots are very popular in traditional medicine in southern Africa to the extent that concern has been expressed about regional extinction. In a survey of the marketing of indigenous medicinal plants in South Africa, *S. aethiopicus* was reported as the ninth most frequently bought plant in the Durban market (Mander, 1997). The rhizomes and roots are traditionally used for coughs, colds and influenza, mild asthma and several other ailments and cultural practices (Hutchings et al., 1996, Steenkamp et al., 2003 and 2005).

Limited scientific investigations have concentrated on *S. aethiopicus* pharmacological activities and its active components, while the plant has been included in several general surveys associating it to diverse biological effects. Antioxidant and radical scavenging activity of extracts of *S. aethiopicus* was reported by Steenkamp et al. (2005), while Lindsey et al. (1999) showed an inhibitory effect of *S. aethiopicus* in the production of prostaglandins that was later confirmed by Jäger and Van Staden (2005) reporting an inhibitory effect of extracts from this plant on COX-1 activity.
Extracts from *S. aethiopicus* have also been associated with anti-infective activity and Lategan et al. (2009) recently published a report of antiplasmodial activity for extracts of *S. aethiopicus*, while Motsei et al. (2003) had previously reported an inhibitory effect on different species of *Candida*. The composition of *S. aethiopicus* extracts has only been partially elucidated and two furanoterpenoids were isolated from a chemotaxonomical investigation of this plant (Holzapfel et al., 2002). The composition of the essential oil obtained through hydrodistillation was also reported by Viljoen et al. (2002).

Although this plant is traditionally used for various respiratory ailments (Steenkamp et al., 2005), no studies have been performed to evaluate its anti-asthmatic properties or pharmacological actions related to its action on respiratory airways. Potential molecular targets of anti-asthmatic or anti-allergic drugs include glucocorticoid receptors and histamine receptors as mediators of inflammation and phosphodiesterases due to their implication in smooth muscle relaxation.

Glucocorticoids are widely used in the treatment of asthma and other allergic diseases, and act through binding to the glucocorticoid receptor (GR), which has a dual action: it up-regulates anti-inflammatory genes by interacting with glucocorticoid response elements and it also suppresses inflammatory genes by interacting with their co-activators (Barnes et al., 1996).

Phosphodiesterases (PDE) are a family of enzymes that metabolize cAMP and cGMP and have received attention as targets for anti-inflammatory and anti-asthmatic therapy (Torphy et al., 1991, Giembycz et al., 1992). Since cyclic nucleotides are
important second messengers in the cells of many tissues and organs, development of therapeutics that selectively target specific PDE isoforms is considered an important goal (Fujii et al., 1998). PDE4 is believed to be the most important PDE isoform in inflammation, particularly allergic inflammation, as it inhibits both eosinophil migration and activation (Torry et al., 1994, Underwood et al., 1993). PDE4 also plays a role in bronchial relaxation and cytokine gene expression (Fujii et al., 1998, Rabe et al., 1995 and Billington et al., 2008), supporting its possible role in asthma.

Histamine is a primary amine released by mast cells and basophils after IgE cross-linking by an allergen, with effects such as vasodilation, mucus hypersecretion, oedema and smooth muscle cell contraction (Marone et al., 2003, White et al., 1990 and Gelfand et al., 2002). It also has pro-inflammatory properties through its effect on cell types such as macrophages, T cells, epithelial and endothelial cells (Gelfand et al., 2002). As most allergic and inflammatory effects of histamine are mediated by binding to H_1 receptors, H_1 receptor antagonists are widely used to treat allergic rhinitis and allergies (White et al., 1990). Cumulative evidence suggests that histamine release may also have a pathophysiologic role in allergic asthma (Gelfand et al., 2002).

Mouse models of allergic airway disease have many of the features of asthma, including airway hyper-reactivity and airway obstruction caused by infiltration of immune cells and mucus hypersecretion (Kips et al., 2003). One of the most common methods for inducing allergic bronchopulmonary inflammation in mice involves systemic sensitisation with a specific antigen and Th-2 skewing adjuvant, usually ovalbumin (OVA) adsorbed onto aluminium hydroxide (Alum), followed by airway
challenge with the same antigen (Reader et al., 2003) or to broncho-constricting agents such as methacholine (Tomkinson et al., 2001).

In this study, we aimed to evaluate the anti-asthmatic properties of *S. aethiopicus* by investigating its effect in glucocorticoid receptor, histamine receptor and PDE IV bioassays and in a mouse model of OVA-induced allergic airway disease.

2. Materials and Methods

2.1 Plant material

The roots and rhizomes of *Siphonochilus aethiopicus* were collected from different sites in South Africa. The voucher specimens were deposited and identified at the South African National Biodiversity Institute (SANBI).

2.2 Extraction methods

The roots and rhizomes were cut into small pieces and dried in an oven at 30-60°C. Dried material was ground to a coarse powder using a hammer mill with a sieve size of 5 mm and stored at ambient temperature prior to extraction.

2.2.1 Diethyl ether extract

500 ml of diethyl ether was added to 300 g of dried, ground rhizomes and left to stand for 1 hour with occasional stirring. The ether was filtered and the residual plant material further extracted overnight with 300 ml diethyl ether followed by filtration. Finally a third extraction of the pulp was done with 300 ml diethyl ether for 1 hour
with filtration. The pulp was finally discarded and the filtrates combined and dried in vacuo using a Buchi rotavapor to give a dried diethyl ether extract of 6.40 g.

2.2.2 Ethanol extract

300 g of dried plant material was extracted with 500 ml of absolute ethanol for 1 hour with occasional stirring and filtered. The pulp was further extracted overnight with 500 ml ethanol and filtered again. Finally a third extraction of the pulp was done with 500 ml ethanol for 1 hour with filtration. The pulp was finally discarded and the filtrates combined and dried in vacuo with a Buchi rotavapor to give a dried ethanol extract of 7.91 g.

2.2.3 Aqueous extract

30 g of dried plant material was weighed out and extracted three times with 500 ml of boiling water for 10 minutes. After extraction, the pulp was filtered through Whatman no.1 filter paper, the filtrates combined and frozen at -20°C. The aqueous extract was concentrated by freeze-drying to yield a freeze-dried extract of 4.57 g.

2.3 Isolation of compound 1

Two hundred milligrams of the diethyl ether extract was subjected to fractionation using flash chromatography on silica gel and eluting with increasing polarity of 5% ethyl acetate/hexane to 100% ethyl acetate to give semi-pure fractions containing compound 1 as the main constituent. Repeated fractionation of the combined fractions using the same solvent system through flash chromatography yielded compound 1 (25 mg). The remaining fractions which lacked the presence of compound 1 were combined into one fraction for bioassaying purposes. The structure
of the furanoterpenoid was confirmed through NMR and mass spectrometry. The $^1$H, $^{13}$C as well as 2-D NMR spectral data (HMQC, HMBC, NOESY) were identical to that published (Holzapfel et al., 2002).

2.4 In vitro biological assays

2.4.1 Phosphodiesterase (PDE IV) enzyme assay

The assay was conducted at MDS Pharma Services in Taiwan. PDE IV partially purified from human U-937 pronocytic cells was used. The extracts and test compound and/or vehicle was dissolved in dimethyl sulphoxide (DMSO) and incubated with 0.2 µg enzyme and 1 µM cAMP containing 0.01 µM [$^3$H]cAMP in Tris buffer pH 7.5 for 20 minutes at 30°C. The reaction was terminated by boiling for 2 minutes and the resulting AMP was converted to adenosine by addition of 10 mg/ml snake venom nucleotidase and further incubation at 30°C for 10 minutes. Unhydrolyzed cAMP was bound to AGI-X2 resin and the remaining [$^3$H]adenosine in the aqueous phase was quantitated by scintillation counting. The extract and compound were dissolved separately in DMSO and tested at concentrations of 1000, 300, 100, 30, 10, 3, 1 and 0.3 µg/ml and 300, 100, 30, 10, 3, 1, 0.3 and 0.1 µg/ml respectively.

2.4.2 Glucocorticoid binding assay

The assay was conducted at MDS Pharma Services in Taiwan and measured binding of [$^3$H]dexamethasone to glucocorticoid receptors in the absence or presence of the plant extracts or purified compound. HeLa S3 (human epitheliald cervix carcinoma) cells expressing the glucocorticoid receptor were suspended in modified HEPES pH 7.2 buffer using standard techniques. Cells (1 x 10^6) were incubated with
6 nM $[^{3}\text{H}]$ dexamethasone for 120 minutes at 25°C. Non-specific binding was estimated in the presence of an excess of 20 µM dexamethasone. Membranes from cells were filtered and washed 3 times and the filters were counted to determine $[^{3}\text{H}]$dexamethasone specifically bound. The extracts and compound were dissolved separately in DMSO and tested at concentrations of 1000, 300, 100, 30, 10, 3, 1 and 0.3 µg/ml and 300, 100, 30, 10, 3, 1, 0.3 and 0.1 µg/ml respectively.

2.4.3 Histamine H$_{1}$ binding assay

The assay was performed at MDS Pharma Services in Taiwan. Membranes from human recombinant histamine H$_{1}$ receptor expressed in CHO cells were used in modified Tris-HCL buffer pH 7.4. A 10 µg aliquot of membranes was incubated with 1.2 nM $[^{3}\text{H}]$pyrilamine for 180 minutes at 25°C. Non-specific binding was estimated in the presence of an excess of 1 µM pyrilamine. Membranes containing receptor proteins were filtered and washed, the filters were then counted to determine $[^{3}\text{H}]$pyrilamine specifically bound. The extract and compound were dissolved separately in DMSO and tested at concentrations of 1000, 300, 100, 30, 10, 3, 1 and 0.3 µg/ml and 300, 100, 30, 10, 3, 1, 0.3 and 0.1 µg/ml respectively.

2.5 Mouse model of allergic airway inflammation

BALB/c mice were sensitized intraperitoneally with 50µg of ovalbumin (OVA) (Sigma) in 1.3% alum adjuvant at day 0, 7 and 14, and challenged intranasally with 1mg of OVA at days 21, 22 and 23. At day 24, airway hyper reactivity was measured by whole body plethysmography (emka Technologies, France) in response to increasing doses of β-methacholine (Sigma, Germany) (Hamelmann et al., 1997). Mice (n = 4-5) were treated intraperitoneally or orally with S. aethiopicus extracts
(ethanol extract, diethyl ether extract, water extract, powdered plant material), the purified furanoterpenoid, dexamethasone, DMSO carrier (Rollins et al., 2006), Tween80 carrier (Polizzi et al., 2000) or TEC (Tween80 /cremaphor/ethanol) carrier (Polizzi et al, 2000), two times daily during the intranasal challenge (day 21-23) and one hour before the methacholine challenge (day 24)(see Table 1). The various extracts were tested in 1-3 independent experiments. On day 25, mice were euthanized and samples were taken for analysis. Protocols were approved by the Research Ethics committee of the University of Cape Town. Mice were housed in individually ventilated cages under specific pathogen free conditions.

2.5.1 Bronchoalveolar lavage

After the mice were euthanized as described previously, lungs were lavaged with 1ml of phosphate buffered saline. Cells were separated onto slides by cytospin, following with a stain using the Rapidiff stain set (Clinical Diagnostics CC, South Africa). For each mouse, 300 cells were counted under a light microscope.

2.5.2 Histology

Lung tissue was preserved in 4% phosphate-buffered formalin for at least 24 hours and then embedded in wax. Paraffin sections of 5μm were cut and fixed to slides, and stained with haemotoxylin and eosin (H&E).

2.5.3 Statistical analyses

Statistical analyses were performed in GraphPad Prism 4.0. (GraphPad software Inc.) using one-way ANOVA with Bonferroni’s post-test.
3. Results

3.1 In vitro activity of *S. aethiopicus* extracts and compound I

*In vitro* bioassays were initiated in order to investigate anti-inflammatory and anti-allergic properties of *S. aethiopicus*. The bioassays or screens were chosen based on targets involved in the activity of products that are currently used to treat allergies and asthma or play an important role in respiratory diseases. To obtain an indication of their activities in these key systems, the diethyl ether extract, the aqueous extract and the purified furanoterpenoid compound of *S. aethiopicus* were evaluated using the glucocorticoid receptor binding assay, histamine receptor binding assay and phosphodiesterase IV inhibition assay at a single concentration and the results are shown in Table 2. As the aqueous extract did not exhibit any activity in these assays, dose response studies were conducted for the diethyl ether extract and the furanoterpenoid for which the results are given in Tables 3 and 4. The diethyl ether extract exhibited good activity in the glucocorticoid receptor binding assay (IC\(_{50}\) of 12.9 µg/ml) as well as the phosphodiesterase IV enzyme assay (IC\(_{50}\) of 26.69 µg/ml). The purified compound also showed similar activity to that of the diethyl ether extract (Table 4). Together these results show that the diethyl extract and purified compound 1 of *S. aethiopicus* have significant activity *in vitro* at systems related to anti-inflammatory and anti-allergic effects *in-vivo*.

3.2 Effect of *S. aethiopicus* extracts on allergic airway disease in mice

In order to test the effectiveness of *S. aethiopicus* against allergic inflammation *in vivo*, mice were sensitized and challenged with the model allergen ovalbumin and concomitantly administered *S. aethiopicus* extracts or the purified furanoterpenoid compound by different methods.
Airway hyperreactivity as measured by whole body plethysmography was increased in ovalbumin-sensitized mice challenged with methacholine compared to naïve controls and did not significantly decrease in any group after administration of *S. aethiopicus* extracts (Figure 2A). In our model, dexamethasone at the dose of (1 mg/kg was also ineffective in reducing metha-choline induced airways hyperresponsiveness. However, both dexamethasone and *S. aethiopicus* extracts demonstrated anti-inflammatory properties in the lung. When administered intraperitonially, the diethyl ether extract of *S. aethiopicus* decreased allergic inflammation in the lung similarly to the dexamethasone control, as seen by a significantly reduced percentage of eosinophils in the bronchiolar lavage fluid (see Figure 2A) and reduced immune cell infiltration around airways and blood vessels (Figure 3). Oral administration of the powdered plant material and diethyl ether or ethanol extract also significantly decreased numbers of eosinophils in the bronchiolar lavage fluid (Figure 2B) and lung inflammation (Figure 3). Neutrophils were present in the bronchiolar lavage fluid in smaller numbers than eosinophils, but were also significantly reduced by intraperitoneal administration of dexamethasone or diethyl ether extract (data not shown).

4. Discussion

Asthma is a chronic inflammatory disease of the lungs, characterized by increased sensitivity to bronchoconstriction that is associated with infiltration of immune cells, mucus hypersecretion and structural remodeling to the airways (Elias et al., 1999). Acute exacerbations, or attacks, may occur after exposure to triggers such as allergens, cigarette smoke, respiratory viruses, environmental pollutants or physical and emotional stress (Bossé et al, 2008). Current therapies include the use of inhaled
corticosteroids to suppress inflammation and prevent attacks, combined with short acting \( \beta_2 \)-adrenocepter agonists (bronchodilators) to relieve the symptoms of an attack (Holgate and Polosa, 2008). Severe cases of asthma exhibit resistance to corticosteroids, and therefore additional therapies remain of interest (Holgate 2008).

In South Africa, the indigenous plant, *S. aethiopicus*, is used by traditional health practitioners to treat symptoms of wheezing of the chest including colds, coughs, influenza, sinus problems and mild asthma. In this study we aimed to investigate potential anti-asthmatic properties of *S. aethiopicus* in vitro and in vivo. Various types of extracts were prepared using the dried rhizomes of the plant and the major constituent, a furanoterpenoid isolated from the diethyl ether extract of the plant. The diethyl ether extract and the purified furanoterpenoid showed activity in the glucocorticoid receptor binding assay, histamine receptor binding assay and phosphodiesterase IV enzyme inhibition. In particular, significant activity was observed for the diethyl ether extract in the phosphodiesterase IV enzyme assay and the glucocorticoid receptor binding assay when tested at a single dose (Table 2). Dose response studies were also completed for the extract, for which most activity was seen in the glucocorticoid binding assay (IC\(_{50}\) =12.9 μg/ml) indicating that the plant may have a similar mode of action to corticosteroids in the treatment of asthma and allergies.

The furanoterpenoid (compound 1), isolated as a major compound from the ether extract, gave an IC\(_{50}\) of 11.4 μg/ml and 56.5 μg/ml in the glucocorticoid and histamine binding assays respectively. Although these activities correspond to active concentrations in the high micromolar range that could be achieved with some
difficulty following the traditional use of the plant, these data suggests that this compound could in fact be responsible for the observed biological activity of the extract. The lower activity observed in the phosphodiesterase IV assay seems to suggest that the diethyl ether extract and compound are not endowed with a significant bronchodilatory activity. Compound 1 is a non-steroidal component which efficiently (albeit with a low potency) acts at the glucocorticoid receptor level with an IC$_{50}$ in the high micromolar range and thus shows a similar mode of action as commercially available steroidal compounds commonly used for the treatment of allergic diseases, such as asthma.

A mouse model of allergic airway disease was used to evaluate the effects of $S. aethiopicus$ in vivo. $S. aethiopicus$ had no effect on airway hyperreactivity at the doses used. Problems with solubility of the extracts were experienced using solvents that were acceptable for administration to live animals. $S. aethiopicus$ is usually administered to patients by traditional health practitioners through inhalation of vapours or as a hot tea or is chewed fresh, which is difficult to implement in a mouse model. Furthermore, dexamethasone also did not significantly reduce airway hyperreactivity at the dose used in this model, despite decreasing cellular infiltration to the lungs. A previously reported study found that higher doses of dexamethasone were needed to block airway hyperreactivity than were needed to block eosinophilia (Birrell et al., 2003), which appears to be the case in our study. Therefore we cannot rule out that higher doses of $S. aethiopicus$ or a longer course of treatment may have an effect on airway hyperreactivity.
Importantly, the pharmacological data in our studies indicate that *S. aethiopicus* has anti-inflammatory properties *in vivo*. Similar to dexamethasone, diethyl ether extracts of *S. aethiopicus* administered intraperitoneally significantly reduced the percentage of eosinophils in the bronchoalveolar lavage fluid. Eosinophils are a hallmark of allergic inflammation and produce reactive oxygen intermediates that are thought to contribute to tissue damage and airway remodelling in the lung (Holgate, 2008). In addition to the decreased percentages of granulocytes in the bronchoalveolar lavage fluid, we also observed less cellular infiltration around airways and blood vessels in the lungs of mice treated with diethyl ether extracts. Oral administration of powdered plant material or the ethanol extracted plant also had anti-inflammatory effects. The use of the powdered plant material could therefore be preferable, as its administration does not require non-biological solvents.

Interestingly, the isolated compound did not appear to reduce pulmonary inflammation or percentages of eosinophils, while in *in vitro* systems the isolated furanoterpenoid compound showed similar, if not higher, levels of activity than the diethylether extract. Difficulties experienced in dissolving the compound in biologically acceptable carriers, may be partially responsible for the observed poor efficacy in an *in vivo* system that could therefore be secondary to poor availability of the compound. Further studies are therefore required to assess whether alternative carriers or delivery systems can improve the efficacy of the purified furanoterpenoid compound *in vivo*.

5. Conclusion
Biological assays conducted on the extracts of *S. aethiopicus* and the purified furanoterpenoid compound showed effects in *in vitro* glucocorticoid receptor binding, phosphodiesterase IV and histamine H₁ assays, indicating that this plant may have anti-inflammatory, anti-allergic and bronchodilatory effects. The extract was subsequently tested *in vivo* mouse model of allergic airway disease and while no functional effect could be measured on airway hyperresponsiveness, a significant anti-inflammatory effect was observed. In conclusion, our results showed that *S. aethiopicus* had anti-inflammatory properties both *in vitro* and *in vivo*, possibly supporting anecdotal accounts of its effectiveness against asthma, sinusitis, colds and flu.

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