

Siphonochilus aethiopicus, a traditional remedy for the treatment of allergic asthma

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ABSTRACT

Asthma is a chronic inflammatory disease of the lungs, characterized by increased sensitivity to bronchoconstriction associated with infiltration of immune cells and mucus hyper secretion. In South Africa, the indigenous plant *Siphonochilus aethiopicus*, is used by traditional health practitioners to treat colds and flu, wheezing of the chest, coughs, influenza, sinus problems and mild asthma. In this study we aimed to investigate the potential anti-inflammatory and immune-modulating properties of *S. aethiopicus* *in vitro*. The dried and powdered *S. aethiopicus* plant material was extracted with organic solvents. The dried extracts were screened *in vitro* in the transcription response, NF- κ B and a cytokine assay. Significant activity was observed for organic extracts of the plant in these assays. This study provides evidence that *S. aethiopicus* has anti-inflammatory and immune-suppressing properties *in vitro*. These findings may support anecdotal accounts of its effectiveness against allergic asthma.

Keywords *Siphonochilus aethiopicus*, Zingiberaceae, medicinal plants, asthma, allergies, herbal remedies, alternative treatment

INTRODUCTION

Although an overall improvement in the general health of the population was seen in the last few years, the prevalence of allergies and asthma has increased. New treatments and specifically alternative treatments are needed for chronic use by asthmatics to improve disease control as available chronic medications are associated with severe side effects. The World Health Organization (WHO) estimated that approximately 235 million people worldwide suffer from asthma (WHO. Asthma. Fact sheet N°307, 2011). Asthma is a chronic, inflammatory disease of the lungs, characterized by an increased sensitivity to various allergens with subsequent bronchoconstriction associated with an infiltration of immune cells and mucus hyper secretion. At present, there is no cure for asthma, and therefore on-going research into novel therapies for asthma, including herbal remedies is necessary. As the aetiology of allergic disorders is complex, multiple biological sites need to be targeted for better control and medicinal plants in singular or multi-herbal formulation (containing multiple active components acting on different sites) have the potential for therapeutic success.

Herbal remedies often do not have the marked negative side-effects that are associated with many allopathic medications and may also be an effective way of decreasing drug resistance (Eichhorn and Efferth, 2012; Ghavami et al., 2011; Khana et al., 2013; Tapsell et al., 2006). This is mainly due to the lower concentration of active molecules that are found in the herbal treatments as compared to single, concentrated active molecules in the pharmaceutical drugs. Therefore, herbal remedies have a milder effect on the physiology of the human body, hit multiple targets (Saller and

Rostock, 2012) with their mixture of components and can be used chronically for longer periods with a more beneficial overall outcome to the patient as a whole.

In South Africa indigenous medicinal plants are used by more than 70% of the population in their health care needs or cultural practices (Van Staden, 2008). Approximately 3,000 species are used by more than 200000 indigenous traditional health practitioners (THPs) (Van Wyk et al., 1997). THPs in South Africa play a crucial role in providing health care to the majority of the population. They are the first health care providers to be consulted in most cases, especially in rural areas and are deeply interwoven into cultural and spiritual life. One of the leads currently being developed from South Africa's traditional use is *Siphonochilus aethiopicus* for the management of asthma and allergies. The plant, *Siphonochilus aethiopicus* (Schweinf.) B.L. Burtt, is a member of the Zingiberaceae family and it is commonly known as *African Ginger*; 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 small berry-like fruits are produced at or near ground level after the flowering period. The freshly cut rhizomes and roots are very popular in traditional medicine in southern Africa. It is used traditionally mainly for mild asthma, colds, influenza and sinus problems (Van Wyk et al., 1997) and the preparations include cold and hot infusions of the rhizomes and roots, steaming of the rhizomes and inhalation of the vapour, and chewing on the fresh rhizomes.

A major mediator of the inflammatory response characteristic of allergic asthma is the chemokine, interleukin-8 (IL-8) which has been shown to be a potent neutrophil chemotactic factor and activator, recruiting neutrophils (Nocker et al., 1996). The increased levels of IL-8 have been observed for non-eosinophilic asthma, in which there are paralleled increased numbers of neutrophils (Gibson et al., 2001). Neutrophils are also present in large amounts in the bronchoalveolar lavage fluid (BALF) of patients with non-infectious exacerbations of asthma (Lamblin et al., 1998), highlighting their importance in allergic

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asthma, as they are the first cells to enter the airway in response to an allergen challenge (Casale et al., 1996; Smith et al., 1992; Teran et al., 1995). Also, various lines of evidence exist linking increased neutrophil and IL-8 levels with allergic asthma (Boschetto et al., 1989; Jacobi et al., 1998; MacDowell and Peters, 2007; Nocker et al., 1996). Although the role of cytokines in the pathogenesis of human diseases is not yet fully understood, assays for cytokines have become a common feature in research and clinical laboratories (Lydyard et al., 2004; O’Gorman and Donnenberg, 2008; Parkin and Cohen, 2001). The HL-60 cell line has been previously used as a model system in assays to screen for cytokine stimulation to determine immune modulating activities (Raduner et al., 2006). The cytokine, IL-8 was used as a target to determine the immune effects of *S. aethiopicus*.

Therefore, experiments were performed testing the levels of IL-8 in response to treatment of HL-60 cells using the organic extracts of the rhizomes of *S. aethiopicus*. This research aims to show preliminary *in vitro* anti-inflammatory and immune modulating activities that supports the traditional use of the plant *S. aethiopicus*.

MATERIALS AND METHODS

Materials

Analytical grade solvents were purchased from Merck and RPMI-1640, Fetal Bovine Serum (FBS) and L-Glutamine were purchased from Whitehead Scientific. Gentamicin and Phorbol 12-Myristate 13-Acetate (PMA) were purchased from Sigma Aldrich. The Cytometric Bead Array™ Human Inflammation Cytometric Bead array (CBA) kit (551811) was purchased from Beckton Dickinson (BD) Biosciences and analysed using BD Biosciences’ equipment and software.

Plant collection

The roots and rhizomes of *Siphonochilus aethiopicus* were collected from Giyani, Limpopo Province in South Africa. A plant specimen was deposited at the South African National Biodiversity Institute (SANBI) and the plant identified as *Siphonochilus aethiopicus* (Schweinf.) B.L. Burt, from the family Zingiberaceae (voucher number PRE 34817).

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.

Diethyl ether extract

500 ml of diethyl ether was added to 300 g of dried, ground rhizomes and left to stand for 1 h 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 h 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 (2.1% w/w).

Ethanol extract

300 g of dried plant material was extracted with 500 ml of absolute ethanol for 1 h 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 h with filtration. The pulp was discarded and the filtrates combined and dried *in vacuo* with a

Buchi rotavapor to give a dried ethanol extract of 7.91 g (2.6% w/w).

Transcription response, NF-κB (human) assay

Human T lymphocytic Jurkat cells, transfected with a response element-lacZ reporter in which transcription of the β-galactosidase gene is directed by the binding site for the NF-κB transcription factor, are used. Test compound and/or vehicle are incubated with the cells (1.5×10^6 /ml) in the presence of 0.5 μM A23187 and 50 ng/ml phorbol 12-myristate 13-acetate (PMA) in RPMI-1640 pH 7.4 at 37°C for 4 h. Test compound-induced β-galactosidase activity is determined by the conversion of fluorescein di-β-D-galactopyranoside (FDG) to fluorescein. Fluorescence intensity is read on SpectroFluor Plus plate reader. A decrease of 50 percent or more ($\geq 50\%$) in fluorescence intensity, relative to 10 μM cyclosporin A, indicates significant inhibitory activity (Lenardo et al., 1989).

Quantification of cytokines with cytometric bead array (CBA)

The HL-60 cell line was obtained from the European Collection of Cell Culture (ECACC) and maintained in suspension at 37°C in 5% carbon dioxide (CO₂) and 100% relative humidity in RPMI-1640 supplemented with 5% fetal bovine serum (FBS), 2 mM L-glutamine and 50 μg/ml gentamicin. Cells were counted and inoculated in a 96-well microtiter plate at plating densities of 7000 to 10000 cells per well and incubated for 24 h. Test samples (50 μl) were added to specific wells at a concentration of 25 μg/ml and incubated for 48 h. PMA (12.5 ng/ml) was used as a cytokine stimulant and added to the wells at 42 h and placed in the incubator for the remaining 6 hrs. Cells without the drug addition served as the control, while the blank contained complete cell culture media without cells. Ethanolic preparations of *Echinacea spp.* and *Siphonochilus aethiopicus* were tested with and without PMA. After 48 h of incubation, the plate was removed and centrifuged for 2 min at 1000 g; supernatants (50 μl) from each well were removed and placed in Eppendorf tubes separately.

The cytokine, IL-8 was detected using the human inflammation Cytometric Bead Array (CBA) kit (551811; BD Biosciences). Tests were performed according to the manufacturer’s instructions available online. The six bead populations are resolved in the red channel of a BD FACSCalibur flow cytometer. For each set of experiments, a standard curve was generated. The results were expressed as pg/ml and then analysed for their relative expression (control versus treated samples). The lower limit for detection for each cytokine was determined as 10 pg/ml.

Statistical analyses

Statistical analyses were performed with GraphPad Prism 4.0. (GraphPad software Inc.) using a one-way ANOVA with Bonferroni’s post-test. All determinations were done in quadruplicate, and the results were reported as mean \pm standard deviation (SD). Graphs were plotted using Origin version 6.0 (Microcal Software, Inc.).

RESULTS

Cellular based transcription response nuclear factor-κB (NF-κB) assay

A significant inhibition activity was observed for the diethyl ether extract with an IC₅₀ of 14.3 μg/mL (n_H 2.24) in the transcription response nuclear factor-κB assay in the absence of cytotoxicity at concentrations up to 100 μg/ml. Cyclosporin A

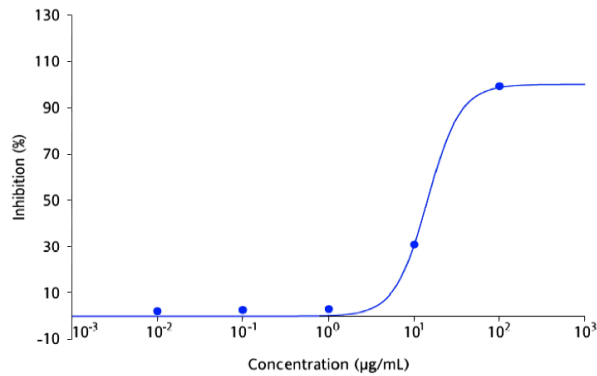


Fig. 1. Effect of the diethyl ether extract of *Siphonochilus aethiopicus* on NF-κB. Concentration response curve in Jurkat cells of NF-κB for diethyl ether extract of *Siphonochilus aethiopicus*. IC₅₀ of 14.3 mg/ml in the absence of cytotoxicity at concentrations up to 100 mg/ml.

was used as the reference compound (IC₅₀ of 0.0608 μM) in this assay. The concentration response curves (Fig. 1) and calculated values (Table 1) are shown below.

Cytokines with cytometric bead array assay

A cytokine expression system using HL-60 cells stimulated with and without PMA was set up to determine the effects of *S. aethiopicus* on the immune system (Faleschini et al., 2012). A positive control (*Echinacea* used as an immune booster) and an ethanol extract of *S. aethiopicus* plant material were tested in the model system using Beckton Dickinson's Cytometric Bead Arrays (CBA) stimulated with PMA (acting as an immune response initiator). The cytokine IL-8 was assayed due to its involvement in the first step of the immune response i.e. inflammation. As shown in Fig. 2 below, PMA stimulated the HL-60 cells to release IL-8 in amounts well above 4000 pg/ml at 12.5 ng/ml. When an extract of *S. aethiopicus* was added to the HL-60 cells with PMA, the expression of IL-8 was completely suppressed (94% inhibition for IL-8).

DISCUSSION

In chronic inflammatory diseases, such as asthma, inflammatory bowel disease and psoriasis, several cytokines recruit activated immune and inflammatory cells to the site of lesions, thereby amplifying and perpetuating the inflammatory state. These activated cells produce many other mediators of inflammation (Epstein et al., 1997). The vicious cycle may be suppressed by glucocorticoid or immunosuppressive therapy, but there is no curative treatment for any chronic inflammatory disease. Transcription factors play a key role in immune and inflammatory responses and one ubiquitous transcription factor of particular importance is NF-κB. NF-κB is a central mediator of the human immune response, regulating the transcription of various pro-inflammatory and inflammatory mediators such as

the cytokines IL-1, -2, -8 and TNF-α, as well as genes encoding cyclo-oxygenase II, nitric oxide synthase, immunoreceptors, cell adhesion molecules, or acute phase proteins (Muller et al., 2004).

In resting cells, cytoplasmic location of the nuclear transcription factor NF-κB is bound by an inhibitory subunit IκB; binding of IκB effectively masks the nuclear localization sequences present on the P50 and P65 subunits of NF-κB, preventing nuclear translocation. Upon cellular stimulation, a signal transduction pathway is activated, leading to phosphorylation of key serine residues in the IκB polypeptide, whereupon the NF-κB-IκB complex dissociates, IκB is rapidly degraded, and the unmasked nuclear localization signal allows NF-κB to translocate into the nuclei and activate the transcription of specific genes. NF-κB represents a master regulator of inflammation and the inhibition of NF-κB could be beneficial in the treatment of asthma and inflammatory diseases. The diethyl extract of *S. aethiopicus* showed significant inhibition in the NF-κB transcription response cellular assay with no cytotoxic effects (IC₅₀ of 14.3 μg/ml). An extract from this plant can thus be used to inhibit specific activity of the NF-κB transcription response, thereby inhibiting the release of various pro-inflammatory and inflammatory mediators that are responsible for the inflammatory pathway of asthma.

The ethanol extract (similar chemical profile to the diethyl ether extract; Fouche et al., 2011) of *S. aethiopicus* was tested for its activity on the release of cytokines with PMA as a co-stimulant on HL-60 cells. For immune cells to work effectively they need to be recruited to the sites of inflammation and appropriately activated. This is achieved by cellular receptors and associated cytokines that bind to these receptors (Lydyard et al., 2004; Parkin and Cohen, 2001). The technique of using stimulated cytokine expression in various immune cells has been widely used. A review article by Spelman et al. (2006) showed that many of the botanical medicines that exhibited immune modulating effects may be due to cytokine modulation. Additionally, cytokine and anti-cytokine immunotherapies have proven to provide valuable therapeutic effects (Zidek et al., 2009). As such,

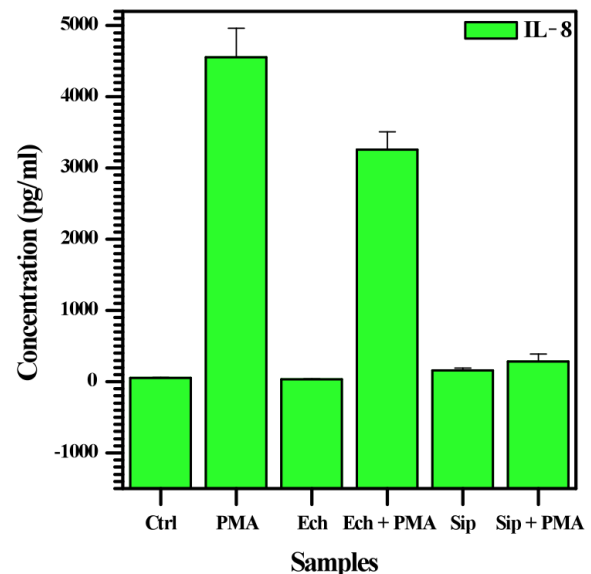


Fig. 2. Effect of the ethanol extract of *Siphonochilus aethiopicus* on IL-8. Concentrations of IL-8 being released by HL-60 cells after a 48 h incubation period with PMA at 12.5 ng/ml, *Echinacea* (Ech) and *S. aethiopicus* (Sip). Statistical analysis n = 4, ± SD; Z-factor = 0.8 - 0.9 (assay prerequisite: Z-factor > 0.5); significant response (decrease of IL-8 concentration by > 50% inhibition).

Table 1. Efficacy and cytotoxicity results of the diethyl ether extract in the NF-κB assay at concentrations up to 100 mg/ml

Concentration	Antagonistic response in cytotoxicity	Antagonistic response in efficacy
100 μM	-4%	141%
10 μM	-2%	42%
1.0 μM	0%	2%
0.1 μM	-1%	1%
10 nM	0%	1%

Note: Cyclosporin A was used as the reference compound; significant response (> 50% inhibition)

modulation of cytokine secretion may offer alternative approaches in the treatment of a variety of diseases in terms of affecting the immune system (Spelman et al., 2006). The cytokine IL-8 was selected due to its role in various pro-inflammatory actions involved in the first step of the immune response i.e. inflammation. An increased release of IL-8 is believed to help the immune system to increase chemotaxis for neutrophils (as well as for T cells and basophils) at the site of inflammation (Lydyard et al., 2004; Parkin and Cohen, 2001). Therefore, decreasing IL-8 concentrations will help to reduce inflammation and thus suppress the increased immune response associated with chronic inflammation. It has been previously reported that IL-8 levels in the body have been found elevated in patients with inflammatory diseases (Harada et al., 1994), pulmonary disease (Sekido et al., 1993), cardiac failure (Kulielka et al., 1995), renal disease (Harada et al., 1994), and urinary tract infections (Ko et al., 1993); indicating that IL-8 is a very sensitive marker in the early stages of inflammation and infection (Sonoda et al., 1997).

The results showed that the ethanol extract of *S. aethiopicus* suppressed the release of IL-8-stimulated by PMA as compared to the positive control (*Echinacea*). *Echinacea* has been known for its immune modulating effects (Spelman et al., 2006; Uluisik and Keskin, 2012). When no PMA was present there was no change in IL-8 levels with the *S. aethiopicus* extract. Further investigations into specificity would be of great value. Botanicals such as *S. aethiopicus* can be employed to suppress an over reactive immune system similar to the drug cyclosporine initially isolated from the fungus *Tolypocladium inflatum* (Borel, 2002). The inhibitory effect of *S. aethiopicus* diethyl ether extract on NF- κ B may well reflect a disruption of IL-8 production, as seen in our experiments. Indeed, the promoter of the IL-8 gene contains binding sites for NF- κ B (Elliot et al., 2001; Kunsch and Rosen 1993; Matsusaka et al., 1993; Mukaida et al., 1994; Oliveira et al., 1994) and NF- κ B has been shown to be central to the regulation of many inflammatory response genes (Grilli et al., 1993; Liao et al., 1994). In addition to the IL-8 gene, NF- κ B also regulates genes encoding other inflammatory cytokines like TNF- α and specific adhesion molecules like vascular cell adhesion molecule-1, mediating the adhesion of specific immune cells like lymphocytes and eosinophils to the vascular endothelium. It has been shown that IL-8 expression, under specific conditions like hypoxia, is mediated through the activation of NF- κ B (Karakurum et al., 1994). Other lines of evidence also link NF- κ B activation to increased IL-8 production (Simone et al., 2011; Yoshida et al., 1998). Research also shows that NF- κ B, which regulates IL-8 expression, is also induced further by this chemokine (Manna and Ramesh, 2005). This inter-relationship of NF- κ B and IL-8 shows that they are closely linked and intimately involved in inflammation and aspects of immunity related to the allergic airway response.

Previous published research on extracts of *S. aethiopicus* (Fouche et al., 2011) demonstrated efficacy in glucocorticoid receptor binding, and 5-lipoxygenase and phosphodiesterase-4 enzyme activities. Reduced infiltration of inflammatory cells in the lung tissue, as well as decreased eosinophils in the bronchiolar lavage fluid in asthmatic rats were also demonstrated for the extract. From the present results *S. aethiopicus* displayed significant suppression of IL-8 with the stimulation of PMA and also significantly inhibited the nuclear transcription factor NF- κ B involved in the regulation of many pro-inflammatory factors. Collectively, these results demonstrated the beneficial properties of the plant extract in the improvement of the symptomatology associated with allergic and infectious respiratory diseases and provide scientific evidence substantiating its traditional use and inclusion in

complementary medicine products. These scientifically validated data substantiate some of the traditional uses of the plant and thus supports significant potential to develop a novel, scientifically proven herbal product with limited unwanted side effects given a long history of use.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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