Anti-inflammatory and antimicrobial profiles of *Scilla nervosa* (Burch.) Jessop (Hyacinthaceae)

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Scilla nervosa (Burch.) Jessop (Hyacinthaceae) [=*Schizocarphus nervosus* (Burch.) Van der Merwe] is a well-known plant in traditional medicine in South Africa, used for conditions associated with pain and inflammation, such as rheumatic fever. However, the topical anti-inflammatory and antimicrobial activities of the plant have not been investigated. A bioassay-guided fractionation approach was implemented to determine the biological activities of different extracts. A crude methanol extract was prepared from the bulbs to investigate the anti-inflammatory properties in a mouse model of acute croton oil-induced auricular contact dermatitis. The non-polar and polar components present in the methanol extract were separated by extraction with dichloromethane and ethanol, respectively; and their antimicrobial activity against the invasive pathogenic microorganisms *Staphylococcus aureus*, *Klebsiellla pneumoniae* and *Candida albicans* was investigated using a microplate method. Oedema induced by application of croton oil was significantly reduced 3 h (~66%) and 6 h (~40%) after treatment with the extracts. Anti-inflammatory activity was ~1.8-fold lower at 6 h, suggesting a potent, short-acting effect. The non-polar extract exhibited greater efficacy and potency against the microorganisms than the polar extract. The non-polar extract was equipotent against *S. aureus* and *K. pneumoniae*, but twice as potent against *C. albicans* as against the bacteria, suggesting little discrimination between Gram-positive and Gram-negative bacteria but specificity for the fungal yeast. The polar extract was the least potent against *K. pneumoniae*, but 10-fold more potent against *C. albicans*, suggesting specificity for Gram-positive bacteria and the fungal yeast. *S. nervosa* contains compounds that are individually, or in combination, potent anti-inflammatory and antimicrobial agents. The anti-inflammatory activity demonstrated here may rationalise the use of the plant in traditional medicine.

Introduction

The skin is a major barrier that protects the body against trauma and invasion by microbial pathogens. To protect the body from the external environment, the skin has many defence mechanisms that are normally closely regulated to suppress inappropriate activity (e.g. psoriasis) and to destroy bacterial (e.g. impetigo and acne) and fungal (e.g. mucocutaneous candidiasis) infections.[1]

Skin disorders are commonly treated with a combination of antibiotics, corticosteroids, antihistamines and moisturisers to reduce itching, infection and inflammation. However, chronic treatment with corticosteroids suppresses lymphocyte function and decreases collagen synthesis, causing skin atrophy and increasing the risk of opportunistic infections. In addition, opportunistic infections may be difficult to treat as a result of acquired resistance against current antimicrobial therapies.[2] Therefore, new therapeutic approaches are being sought, in which not only the inflammatory response is effectively suppressed, but also the causative microorganisms.

Scilla nervosa (S. nervosa) (Burch.) Jessop (Hyacinthaceae) or Wild squill is also known as *Schizocarphus nervosus* (Burch.) Van der Merwe, in isiZulu as *Ingcino* and in Afrikaans as *Blouberglelie*. It is indigenous to Botswana but has naturalised in the grasslands of southern Africa.[3] Bulbs are used in traditional medicine by the Tswana people to treat infertility in women, by the Sotho people to treat gall sickness in livestock[4] and constipation,[5] dysentery and nervous conditions in children,[6] by the Zulu people to treat pain in rheumatic fever, which primarily affects children between the ages of 5 and 15,[5] and by the Xhosa people to treat dysentery and nervous conditions in children.[3] At least one traditional use indicates possible anti-inflammatory properties of the bulbs: treatment of pain in rheumatic fever. However, there have been no reports on the topical anti-inflammatory activity *in vivo* to rationalise its traditional use and antimicrobial activity *in vitro* to probe additional medicinal properties. We therefore investigated these activities.

Materials and methods

Chemicals

Acetone, ethanol, dichloromethane and methanol were obtained from Merck (Darmstadt, Germany). Amphotericin B, neomycin solution, croton oil, diclofenac and dimethylsulphoxide

were obtained from Sigma (St Louis, MO, USA). *p*-Iodonitrotetrazolium chloride violet, Mueller-Hinton broth and Sabouraud dextrose broth were obtained from Fluka (Buchs, Switzerland).

Preparation of extracts from *S. nervosa*

S. nervosa was bought in June 2008 from the Fig Tree Indigenous Nursery (Westville, Durban, South Africa). The plants were identified and a voucher (Du Toit2, NU) was lodged at the University of KwaZulu-Natal Herbarium (Pietermaritzburg, South Africa). All of the plants were of the same developmental stage. The fresh bulbs (1.5 kg) were chopped into small pieces and dried overnight. A bioassay-guided fractionation approach was followed to investigate the biological activities of different extracts. Pieces were soaked in methanol for 3 days, filtered and dried in a rotary evaporator (Heidolph, Darmstadt, Germany). The crude methanol extract (42.3 g), containing polar and non-polar components, was used for the anti-inflammatory assays and to prepare two additional extracts for the antimicrobial assays. Two amounts of 1 g of the crude methanol extract were sonicated for 1 h in equal volumes (20 mL) of dichloromethane or ethanol to separate the non-polar and polar components, respectively. The two extracts were filtered and dried in the evaporator to yield a methanol-dichloromethane and methanol-ethanol extract. Extracts were stored at -80 °C until further use.

Assessment of croton oil-induced oedema

Ethical approval (058/09/Animal) from the University of KwaZulu-Natal Animal Ethics Subcommittee was obtained prior to the investigation of croton oil-induced oedema[7] in a mouse model. Guidelines by the University of KwaZulu-Natal Animal Ethics Subcommittee and Biomedical Resources Unit for the maintenance and treatment of laboratory animals were followed.

This topical study design was based on previous studies where the inhibition of croton oil-induced auricular oedema by emu oil was investigated to screen for agents against contact dermatitis[8,9] and to assess the anti-inflammatory profiles of flavonoids.[10] Briefly, 20 8-week-old male Balb/c mice, weighing approximately 30 g each, were used. The mice were divided into two groups of 10 mice each (two per condition). One group was examined 3 h after treatment and the other group 6 h after treatment. Equal volumes of croton oil and acetone (as a vehicle) were mixed and 50 µl of the mixture was applied to the inner surface of the right auricle of each mouse to induce oedema. The left ears were untreated. Acetone has not been documented to have an anti-inflammatory effect by itself.[1,11] After 1 h, different amounts of the crude methanol extract in acetone were applied (0 mg (control)), 0.1 mg, 0.5 mg and 1 mg in 50 µL) to the right auricle (~1 cm²) and any reduction in oedema was assessed 3 h and 6 h after extract application. Mice were euthanised after 3 h or 6 h, and left and right auricle biopsy specimens were obtained using a 6-mm biopsy punch. The specimens were weighed to assess any differences in oedema.

The non-steroidal anti-inflammatory drug diclofenac in acetone (0.1 mg in 50 µl) was included as a positive control.

Assessment of microbial susceptibility

Mueller-Hinton broth was inoculated with *Staphylococcus aureus* or *Klebsiella pneumoniae* and grown in an incubator at 37 °C, with an optical density of 0.8 at 490 nm. Sabouraud dextrose broth was inoculated with *Candida albicans* and grown in an incubator at 37 °C, with an optical density of 0.5 at 600 nm. *S. aureus* (strain 12600), *K. pneumoniae* (strain 13883) and *C. albicans* (strain 10231) cultures were obtained from American Type Culture Collection (ATCC) (Manassas, VA, USA). The broths were prepared according to the manufacturer's protocol.

The microbial susceptibility assay was based on a microplate method[10,12] but with modifications. The two extracts were prepared in pure dimethylsulphoxide at concentrations of 0.01 mg/mL, 0.05 mg/mL, 0.1 mg/mL, 0.5 mg/mL, 1 mg/mL, 2 mg/mL and 3 mg/mL. Firstly, 100 µl/well of sterile broth was added to a clear, sterile 96-well microtitre plate (Corning Life Sciences, Acton, MA, USA). Secondly, 6 µL/well of the extract was added and the plate tapped. Thirdly, 94 µL/well of sterile water was added and the plate tapped. Finally, 100 µL/well of the appropriate microbial culture was added and the plate was tapped to mix the contents and then incubated at 37 °C for 18 h. Therefore, with a final volume of 300 µL/well and a dilution factor of 50x, the final concentration of dimethylsulphoxide/well was 2% v/v and the final concentrations of the extracts/well were 0.2 µg/mL, 1 µg/mL, 2 µg/mL, 10 µg/mL, 20 µg/mL, 40 µg/mL and 60 µg/mL. Growth of the microorganisms was not significantly inhibited by the 2% v/v dimethylsulphoxide (data not shown). The positive controls were neomycin against *S. aureus* and *K. pneumoniae*, and amphotericin B against *C. albicans* (100 µL at a concentration of 300 µM was added in step 2 above, therefore with a final volume of 300 µL/well and a dilution factor of 3x, the final concentrations were 100 µM). For *S. aureus* and *K. pneumoniae*, 40 µL/well of *p*-iodonitrotetrazolium chloride violet (400 µg/mL in water) was added and the plate tapped and incubated at 37 °C for 15 min. Microbial growth was quantified by colourimetry (490 nm) for bacteria and optical density (600 nm) for *C. albicans* in a microplate reader (BioTek ELx800, Winooski, VT, USA).
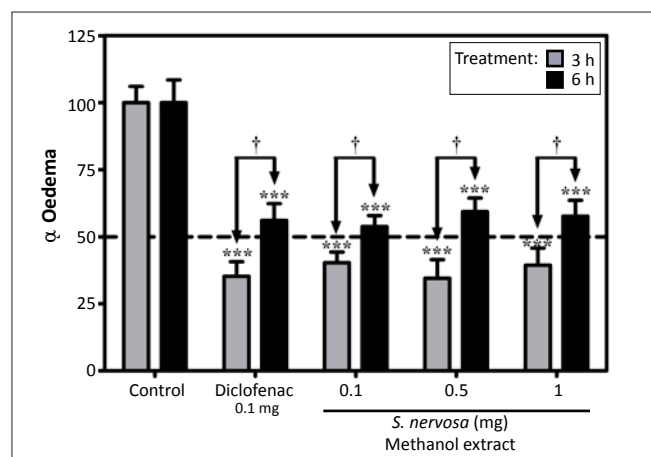
Data analysis

Data are reported as the mean ± standard error of the mean of five (anti-inflammatory assays) and four to six (antimicrobial assays) independent experiments with duplicate measurements per condition. For the anti-inflammatory assays, two mice per each condition (representing the duplicate measurement) were used. Oedema was quantified by calculating the difference in mass of the right and left auricle biopsy specimens and expressed as a percentage of the croton oil control. For the antimicrobial assays, microbial growth was quantified as a percentage of the control without any plant extract. Two wells per each condition (representing the duplicate measurement) were used. GraphPad Prism[13] was used to determine the IC_{50} value (the concentration of

an inhibitor at which a response is reduced by 50%) through non-linear least squares regression. Minimum inhibitory concentrations (MICs) were deduced from the graphs. Statistical comparisons were made by one-way analysis of variance (ANOVA) followed by Bonferroni's post-test or by Student's two-tailed paired t-test to determine p-values. A value of $p < 0.05$ was considered significant.

Results and discussion
Reduction of croton oil-induced oedema

The anti-inflammatory activity of $S.$ $nervosa$ extract in a mouse model of acute croton oil-induced oedema has not been previously studied. Increased skin thickening as a result of oedema is often the first sign of skin irritation and local inflammation. Oedema is one indicator of the complex processes that occur during skin inflammation, including increased vascular permeability, swelling within the dermis and proliferation of epidermal keratinocytes. Ear oedema was measured in the auricular skin at 3 h and 6 h following treatment with the extract to determine sustained anti-inflammatory activity. Topical application of the crude methanol extract in acetone at three different doses significantly inhibited the croton oil-induced increase in skin weight after 3 h (~3-fold) and 6 h (~1.8-fold), indicating the therapeutic ability of $S.$ $nervosa$ to ameliorate croton oil-induced contact dermatitis (Figure 1). This was supported by observed reductions in skin thickness, weight and redness. The greatest anti-inflammatory effects were observed with 0.5 mg at 3 h (inhibition of oedema was 65.5 ± 7.0%) and 0.1 mg at 6 h treatment (inhibition of oedema was 46.2 ± 4.1%). Anti-inflammatory effects of each dose of the extract were significantly lower (~1.8-fold) at 6 h post-treatment than at 3 h, suggesting that $S.$ $nervosa$ is an anti-inflammatory agent with a relatively short duration of action, similar to that of the diclofenac control. Diclofenac at 0.1 mg significantly inhibited oedema after 3 h (64.8 ± 5.4%) and 6 h (43.9 ± 4.1%) treatment. No mice died during the study, nor were any adverse effects observed with the different doses of extract. These outcomes suggest that $S.$ $nervosa$ was non-toxic in mice up to a topical dose of 1 mg/cm^2.

***, $p < 0.001$ versus croton oil control; †, $p < 0.05$ for 3 h versus 6 h after treatment.

FIGURE 1: Anti-inflammatory activity, shown as a percentage of oedema (mean ± s.e.m.), of $Scilla$ $nervosa$ extract, diclofenac and the control (no treatment).

The mechanism of the observed anti-inflammatory activity is unknown. It would be worthwhile to determine the expression of cytokines, such as interleukins (IL) and tumour necrosis factor (TNF), as well as myeloperoxidase and cyclo-oxygenase activities. In a study on the anti-inflammatory effects of an $Asparagus$ $cochinchinensis$ extract, Lee and co-workers[1] demonstrated a significant reduction in mice ear thickness and weight 6 h after treatment. Although the components of the ethanolic extract were unknown, mechanistic studies indicated a reduction in the levels of IL-1β, TNF-α and myeloperoxidase activity. A study by Oliveira de Melo and co-workers[14] on different extracts of $Nectandra$ $falcifolia$ also demonstrated reduced myeloperoxidase activity.

Antimicrobial activity

Microbial skin infections often cause local inflammation as part of the immune response. Because it is demonstrated here that bulbs of $S.$ $nervosa$ possess anti-inflammatory activity, the susceptibility of the important invasive Gram-positive $S.$ $aureus$, Gram-negative $K.$ $pneumoniae$ and the fungal yeast $C.$ $albicans$ to two different extracts prepared from $S.$ $nervosa$ was subsequently assessed. These microorganisms are implicated in skin conditions such as boils, impetigo, acne, mucocutaneous candidiasis and opportunistic infections in immunocompromised patients.
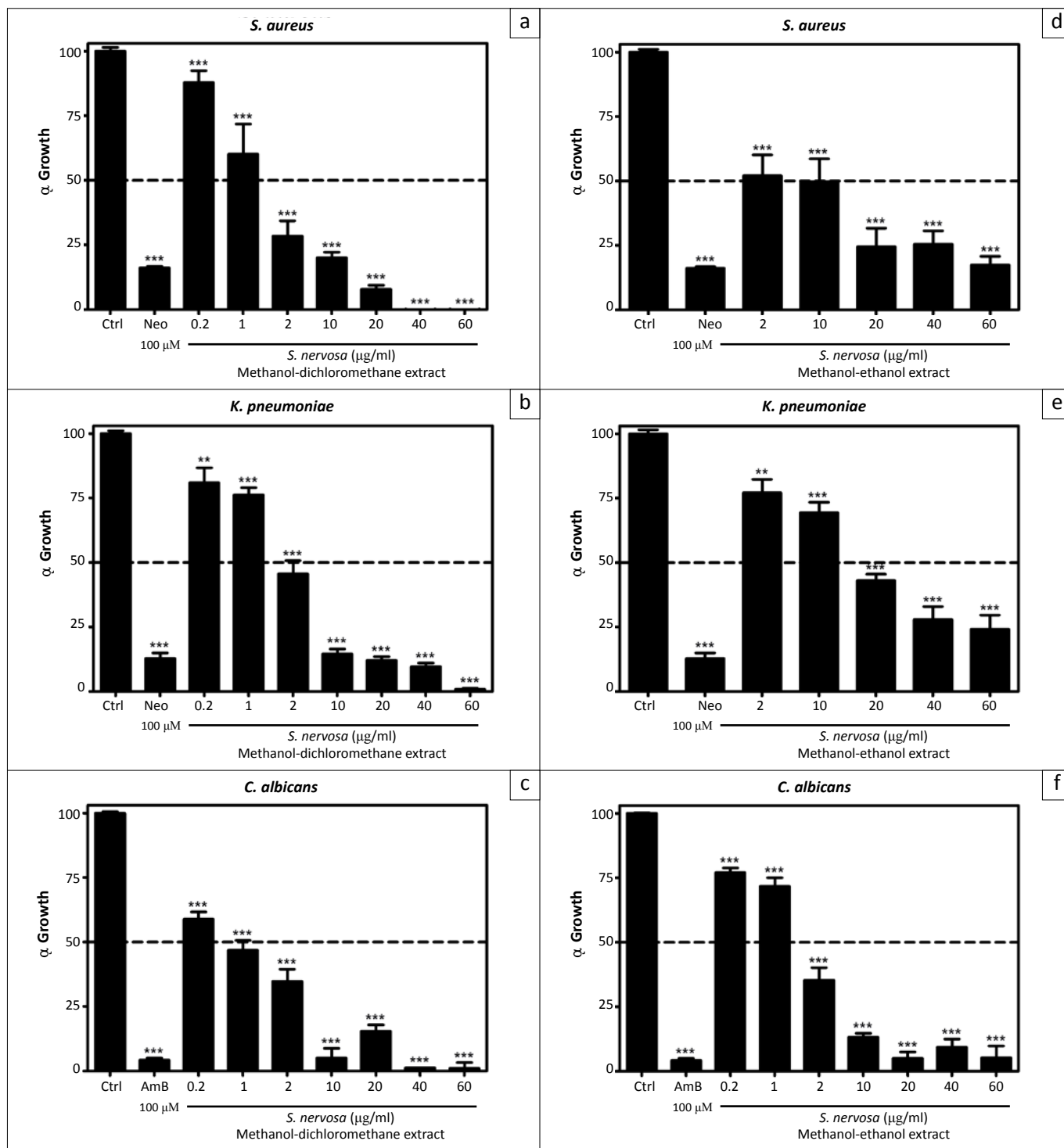
Plant extracts prepared from different solvents contain different ingredients in different ratios. We therefore prepared two extracts: a methanol-dichloromethane extract and a methanol-ethanol extract. The positive control neomycin, at 100 µM, significantly inhibited the growth of $S.$ $aureus$ (83.8 ± 0.6%) (Figure 2a and 2d) and $K.$ $pneumoniae$ (87.2 ± 2.1%) (Figure 2b and 2e), whilst amphotericin B, at 100 µM, significantly inhibited the growth of $C.$ $albicans$ (95.8 ± 0.8%) (Figure 2c and 2f).

Methanol–dichloromethane extract

Growth of $S.$ $aureus$ (Figure 2a), $K.$ $pneumoniae$ (Figure 2b) and $C.$ $albicans$ (Figure 2c) was inhibited in a dose-dependent manner after treatment with the methanol–dichloromethane extract; and total suppression was reached at higher concentrations, suggesting bactericidal and fungicidal activity. The average MIC values were calculated as 40 µg/mL, 60 µg/mL and 40 µg/mL and the average IC$_{50}$ values were calculated as 1.8 ± 0.6 µg/mL, 2.0 ± 0.4 µg/mL and 1.0 ± 0.4 µg/mL for $S.$ $aureus$, $K.$ $pneumoniae$ and $C.$ $albicans$, respectively. This non-polar extract showed equivalent activities against $S.$ $aureus$ and $C.$ $albicans$ and was the least active against $K.$ $pneumoniae$. However, it was almost twice as potent against $C.$ $albicans$ (IC$_{50}$ ratio of ~2) when compared to $S.$ $aureus$ and $K.$ $pneumoniae$, which exhibited similar potencies.

Methanol–ethanol extract

Growth of $S.$ $aureus$ (Figure 2d), $K.$ $pneumoniae$ (Figure 2e) and $C.$ $albicans$ (Figure 2f) was inhibited in a dose-dependent manner after treatment with the methanol–ethanol extract. Average MIC values could not be deduced because total

Ctrl, control (received no treatment); Neo, neomycin (an antibacterial) and AmB, amphotericin B (an antifungal) were positive controls.
**, $p < 0.01$; ***, $p < 0.001$ versus control (no extract).

FIGURE 2: Antimicrobial activity, shown as a percentage of growth (mean ± s.e.m.) of *Scilla nervosa* extracts, methanol-dichloromethane (a–c) and methanol-ethanol (d–f), against the invasive pathogenic microorganisms *Staphylococcus aureus* (a & d), *Klebsiella pneumoniae* (b & e) and *Candida albicans* (c & f).

growth suppression could not be reached even at higher doses (data not shown), suggesting bacteriostatic and fungistatic activity. The general trend observed was that the polar extract was the least active against *K. pneumoniae* and the most active against *C. albicans*. The average IC_{50} values were calculated as 6.2 ± 3.6 µg/mL, 16.2 ± 4.0 µg/mL and 1.6 ± 0.4 µg/mL for *S. aureus*, *K. pneumoniae* and *C. albicans*, respectively. This polar extract was almost 10-fold more potent against *C. albicans* when compared to *K. pneumoniae*

(IC_{50} ratio of ~10) and almost 4-fold more potent when compared to *S. aureus* (IC_{50} ratio of ~3.9). Furthermore, it was more than 2.5-fold more potent against *S. aureus* than *K. pneumoniae*, against which it was the least potent (IC_{50} ratio of ~2.6).

These findings suggest that there may be little difference in activity of the non-polar extract against Gram-positive and Gram-negative bacterial species. The polar extract exhibited

specificity towards Gram-positive bacterial species; both extracts exhibited specificity towards the fungal yeast species, but the non-polar extract was the more potent and had the higher efficacy of the two extracts. These extracts contain different compounds (homoisoflavanones and/or stilbenes) in different ratios.[5] It is possible for these compounds to potentiate each other's activities at different concentrations to dictate microbicidal or microbiostatic activity. In a review by Van Vuuren[15] on the antimicrobial activity of South African medicinal plants, it was suggested that the identification of a single active component responsible for activity is becoming more improbable and that research should be focusing on the investigation of a combination of compounds to achieve greater efficacy. Based on our findings, it is clear that the non-polar extract was more potent against the microorganisms than the polar extract. Future aims are to determine the specific compounds present in each extract as well as their concentrations, and to determine individual activity as well as their combined activity.

Conclusion

Our findings suggest that extracts from *S. nervosa* bulbs may be important in the treatment of not only idiopathic inflammatory skin diseases, but also bacterial and fungal skin diseases. Furthermore, our findings rationalise the traditional use of the plant as an anti-inflammatory agent and may be useful towards the development of new anti-inflammatory and antimicrobial therapies with specificity towards certain microbes. Finally, it will be worthwhile to characterise the various mechanisms of the anti-inflammatory activity.

Acknowledgements

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