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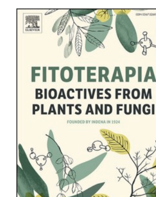

Exploring indigenous South African plants as alternative treatments for dermatophytosis: Focusing on the antifungal properties and mechanism of action of *Searsia lancea*

Murunwa Madzinga [a], Mammoloro Boitshoko L. Malefo [a], Chris van der Merwe [a], Marco Nuno De Canha [a], Ashish Wadhwani [b,c], Namrita Lall [a], Quenton Kritzinger [a,*]

[a] Department of Plant and Soil Sciences, University of Pretoria, Private Bag X20, 0028, South Africa
[b] Department of Pharmaceutical Biotechnology, JSS College of Pharmacy, JSS Academy of Higher Education and Research, Ooty 643001, India
[c] Faculty of Health Sciences, School of Pharmacy, JSS Academy of Higher Education and Research, Vacoas 73304, Mauritius

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ABSTRACT

Numerous medicinal plants are reported to have activity against dermatophytes, however, there are limited studies providing insights into their mechanism of action, which may be hindering their clinical use. This study aimed to investigate the antifungal activity and toxicity of three South African plants traditionally used to treat skin infections caused by dermatophytes and to investigate the mechanism of action of the most active plant extract. *Searsia lancea* showed the highest antifungal activity against *Microsporum canis* (MIC 0.156 mg/mL). *Warburgia salutaris* and *M. comosus* showed no toxic effects on HaCaT cells while *S. lancea* exhibited moderate cytotoxicity. The most active combination of *S. lancea* combined with *M. comosus* showed to be non-toxic. *Searsia lancea* and *M. comosus* were non-mutagenic at 500 μg/mL. The ethyl acetate partition of *S. lancea* demonstrated a two-fold increase in activity against *Microsporum* species while fraction fifteen (F15) exhibited a four-fold increase in activity against *T. mentagrophytes*. Two compounds in F15 were identified as sakuranetin and gentisic acid, with sakuranetin showing the best activity against *T. mentagrophytes*. Electron microscopy showed alterations of hyphal surfaces in the form of shrinkage and folding of the plasma membrane (24–48 h) and breakage and leakage of cytoplasmic material (72 h). The RT-qPCR showed significant repression ($p < 0.01$) of the *SSU1* gene of *M. canis* treated with *S. lancea* (0.312 mg/mL) after 2 and 7 days. The findings not only support traditional usage of *S. lancea* but also provide targets of *S. lancea*'s anti-dermatophytic activity.

1. Introduction

Dermatophytes are pathogenic fungi that degrade keratin [1]. They are responsible for the most prevalent skin infections worldwide [2,3]. These parasitic fungi grow mainly on the outermost layer of the skin (the epidermis), affecting the skin, hair and nails [1,4,5].

Dermatophytes may cause serious infections in immunocompromised and immuno-suppressed individuals [5]. The prevalence of dermatophytosis has increased with increasing cases of individuals with Human Immunodeficiency Virus and Acquired Immunodeficiency Syndrome (HIV/AIDS), diabetic patients and those who have undergone organ transplants [6]. South Africa has one of the highest incidences of HIV and has more than two and a half million diagnosed diabetics [7–9].

Several topical and oral medications are used in treating dermatophytosis, including griseofulvin and clotrimazole [10–12]. Topical and oral antifungal treatments are costly and require long-term regimes, which often result in affected individuals discontinuing treatment, which often leads to the recurrence of infections and contributes to the burden of antifungal resistance in some species of dermatophytes [13]. Antifungal active ingredients often exert a toxic effect on mammalian cells due to shared similarities between the eukaryotic cell structures and provide antifungal drugs with limited cellular targets specific to the fungi [14–16]. Therefore, there is an urgent need to search for new, effective and 'improved' molecules.

According to the World Health Organization (WHO), 80 % of people in developing countries are reliant on plants as their primary healthcare

* Corresponding author at: Department of Plant and Soil Sciences, University of Pretoria, Pretoria 0028, South Africa.
E-mail addresses: murunwa.madzinga@tuks.co.za (M. Madzinga), malefom@arc.agric.za (M.B.L. Malefo), u28349505@tuks.co.za (M.N. De Canha), dradwadhwani@jssuni.edu.in (A. Wadhwani), namrita.lall@up.ac.za (N. Lall), quenton.kritzinger@up.ac.za (Q. Kritzinger).

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resource [17,18]. Medicinal plants and extracts thereof are commonly used to treat skin infections in rural areas and are highly sought after in the treatment of dermatological disorders as they are perceived to have the ability to speed up healing, stop bleeding and alleviate other skin conditions [3,17].

South Africa is rich in botanical diversity with over 30,000 indigenous plant species [19–21]. Approximately 3000 of these indigenous species are documented and used medicinally [20,21]. Southern African plants such as *Dicoma anomala* Sond (roots, stems and leaves) and *Ekebergia capensis* Sparrm (bark) have been used to treat various skin ailments [15,17]. Despite the large number of plants being traditionally used to treat different ailments, research on medicinal plants as dermatological treatments has not received the necessary attention it deserves in the southern parts of Africa [17,22,23].

Several plant species such as *Aristea ecklonii* Baker, *Calpurnia aurea* (Aiton) Benth. subsp. *aurea* and *Carica papaya* L., with scientifically proven antifungal activity against dermatophytes, have been reported [24–26], however, limited information on their mechanism of action (MoA) is available [27]. Knowledge of the MoA of any therapeutic agent enables better dosing, by allowing researchers to monitor the effects of the agent on the targeted pathway and also assists scientists in designing clinical trials [28]. In addition, it may provide information on the safety of the 'drug' and how it may affect the body [29]. Plant extracts are believed to have multiple mechanisms of action which reduce drug resistance [30].

In this study, the leaves of *Melianthus comosus* Vahl. (Honey flower shrub) and *Warburgia salutaris* (G. Bertol.) Chiov (Pepper-bark tree) and the underexplored species, *Searsia lancea* (L.f.) F.A. Barkley (Karee tree) were selected based on both documented pharmacological evidence, ethnobotanical knowledge and traditional usage. The leaf material of *S. lancea* and the bark of *W. salutaris* have been used by Vhavenda people as herbal washes for the treatment of skin diseases in animals [31,32]. The leaves of *S. lancea* have been used for the treatment of animal skin infections while the bark and leaves of *W. salutaris* have been used to treat ringworm. *Melianthus comosus* has been documented for its use in southern Africa for the treatment of septic wounds [33]. Although numerous studies have reported on the antifungal activity of the leaf extracts of the selected plants and the metabolites responsible for their antimicrobial activity [24,34–36], no studies have focused on their antifungal activity by determining their MoA, particularly for *S. lancea.*

The primary aim of this study was to validate the use of three selected South African indigenous plants for dermatophytic skin infections by investigating their antifungal activity against three dermatophytes. In addition, the safety of the extracts on skin cells and their effects on deoxyribonucleic acid (DNA) were evaluated, and the potential antifungal biomarkers of the most active extract, *S. lancea* were determined. Furthermore, the antifungal MoA of this plant was evaluated against *M. canis*.

2. Materials and methods

2.1. Preparation of plant extracts

The leaves of mature *S. lancea* (PRU0124367)*, W. salutaris* (PRU0123558) and *M. comosus* (PRU0124368) were collected in February 2020, from the Manie van der Schijff Botanical Garden, University of Pretoria, Pretoria, South Africa. Specimens were collected and deposited at the H.G.W.J. Schweickerdt Herbarium, University of Pretoria where a voucher specimen number was deposited. The collected material was air-dried in the shade at room temperature for four weeks, and thereafter, ground to a coarse powder using an IKA grinder (MF 10) equipped with a 4.0 mm sieve (United Scientific, South Africa). The powered leaves (200 g) were macerated in 2 L of 96 % ethanol and placed on a Labcon 3086 U (Labotec, South Africa) orbital shaker for 72 h. The macerations were then filtered using a Buchner funnel and concentrated using a BUCHI Rotavapor B-480 (Buchi, Switzerland). The ethanolic extracts were then dried using an Alpha 1–2 LD plus freeze-dryer (Lasec, South Africa). The crude extracts were stored in closed vials at 4 °C until further use.

2.2. Anti-dermatophytic screening

2.2.1. Preparation of fungal culture and stock solutions

Microsporum canis (ATCC 36299) and *Microsporum gypseum* (clinical isolate) were grown and maintained on potato dextrose agar (PDA) while malt extract agar (MEA) was used for *Trichophyton mentagrophytes* (ATCC 9533). All pathogens were sub-cultured onto MEA plates and incubated at 25 ± 2 °C for 3 weeks. For the antifungal activity, the fungal test organisms were transferred to malt extract broth (MEB) and the optical density was adjusted to attain a 0.5 McFarland standard (5×10^6 CFU/mL) [37].

Thirty milligrams (30 mg) of plant extracts were dissolved in 10 % dimethyl sulfoxide (DMSO) (in malt extract broth). Griseofulvin was used as a positive control and prepared to a stock concentration of 0.6 mg/mL.

2.2.2. Antifungal activity of crude extracts

The broth microdilution dilution assay was used to screen the activity of the plant extracts against the dermatophytes according to [38] with minor modifications. Aseptically, 100 μL of MEB was added to all the wells of a 96-well plate. One hundred microlitres of each plant extract (30 mg/mL) was added to the first row of the plate in quadruplicates, followed by a 2-fold serial dilution. Inoculation was carried out by transferring 100 μL of fungal suspension (5×10^6 CFU/mL) to all the sample wells. The fourth column of each plant extract was used as a colour reference. Controls included the positive (griseofulvin), viability (fungi only), medium sterility (MEB only), and negative (2.5 % *v/v* DMSO). Sealed microtitre plates were incubated at 25 ± 2 °C for 72 h. Thereafter, 40 μL of 0.6 mg/mL solution of iodonitrotetrazolium chloride (INT) was added to all the wells of the 96-well plate followed by incubation for 2–24 h, depending on the fungal isolate and formazan crystal formation. The minimum inhibitory concentration (MIC) was determined as the lowest concentration that visibly contained no formazan crystals. The plant extract concentrations ranged from 0.059 to 7.50 mg/mL, while that of the positive control, griseofulvin, ranged between 0.002 and 0.150 mg/mL. Three independent experiments were performed with each trial containing triplicates of the tested extracts.

2.2.3. Interactive combination studies

The plant extracts were tested in three combinations to determine whether combinations would exhibit reduced toxicity and improved antifungal efficacy. The combinations of plant extracts were as follows: *S. lancea* and *M. comosus; S. lancea* and *W. salutaris*; *M. comosus* and *W. salutaris* which were tested at different ratios (9:1; 8:2: 7:3; 6:4; 5:5: 4:6: 3:7: 2:8 and 1:9). The antifungal screening was carried out using the broth microdilution assay as outlined in 2.2.2. The results were analysed using Microsoft Excel and the interactions between the plant extracts were classified using combinational indexes (CI) determined by the sum of fractional inhibitory concentrations ($\sum$ FIC) calculated using the formula below as described by [24,39,40].

$$CI = \sum FIC$$

$$\sum FIC = FIC^{i} + FIC^{ii}$$

$$FIC^{i} = \frac{MIC\ of\ A\ in\ combination\ with\ B}{MIC\ of A\ independently}; FIC^{ii} = \frac{MIC\ B\ in\ combination\ with\ A}{MIC\ of\ B\ independently}$$

The combinational indexes were interpreted as synergistic (CI ≤ 0.5), additive (0.5 < CI ≤1.00), indifferent/ no interaction (1.0 < CI ≤

4.0) and antagonistic (CI >4.0) [24,40].

The experiment was conducted in three independent replicates and each independent experiment was performed in triplicate.

2.3. In vitro antiproliferative activity

Immortalised human keratinocyte cells (HaCaT) were maintained in T-75 flasks with Dulbecco's Modified Eagle's Medium supplemented with 1 % of antibiotics (penicillin (100 U/mL), streptomycin (100 μg/mL) and amphotericin B (25 μg/mL)) and 10 % heat-inactivated fetal bovine serum. The cells were incubated at 37 °C in a humidified environment supplemented with 5 % carbon dioxide and maintained until 80 % confluency before plating.

The methoxynitrosulfophenyl-tetrazolium carboxanilide (XTT) cell proliferation kit II (Roche Applied Sciences, South Africa) was used to determine the antiproliferative activity of the plant extracts on HaCaT cells as described by [41]. Stock solutions of 2 mg/mL of the three ethanolic extracts were prepared and serial diluted in 24-well plates. The resulting concentrations of the individual extracts and a combination of *S. lancea* and *M. comosus* (ratio 9:1) were evaluated for their antiproliferative effects.

One hundred microlitres of cell suspension (100,000 cells/mL) was transferred to all the inner wells of the 96-well microtitre plate and incubated for 24 h under the conditions described above. Following attachment of the cells, 100 μL of the diluted plant extract was added to respective wells to achieve concentrations ranging from 3.125 to 400.0 μg/mL, and 3.9×10^{-4}–0.05 μg/mL for the positive control (Actinomycin D). Colour control (samples without cells), untreated cell control and a 2 % *v*/v DMSO vehicle control were included. The plates were incubated for a further 72 h and thereafter, 50 μL of XTT cell viability reagent was added to all the wells and plates were incubated for an additional 2 h before reading. Absorbance readings were recorded at 570 nm with a 600 nm reference wavelength, using a BIOTEK Wave XS multi-well plate reader (Analytical & Diagnostic Products CC, South Africa). The IC_{50} values were calculated using GraphPad Prism 4.0 software using percentage inhibition values calculated using the equation below.

$$\%Cell\ viability = \frac{sample\ absorbance}{control\ absorbance} x\ 100$$

where sample absorbance is the absorbance of [(Cell suspension + tested extract and XTT) – (sample + XTT)] and the control absorbance is the absorbance of [(XTT + solvent vehicle control) – (values of solvent vehicle control)] [42].

The IC_{50} values were calculated from the dose-response curves using non-linear regression analysis. The obtained IC_{50} were subjected to a one-way analysis of variance (ANOVA) followed by Dunett's multiple comparison test comparing samples with the positive control. Data obtained is expressed as mean ± standard deviation of at least three independent replicates ($n = 3$) with each independent experiment with samples tested in triplicate.

The toxicity was classified according to the guidelines by the United States National Cancer Institute, which state that crude extracts are classified as significantly toxic ($IC_{50} < 30$ μg/mL), moderately toxic ($30 < IC_{50} \leq 100$ μg/mL), and non-toxic ($IC_{50} > 100$ mg/mL) [43,44].

2.4. Mutagenicity test

Mutagenicity was carried out on the three ethanolic extracts using the Ames test kit (Medox Biotech, India) following the manufacturer's instructions. The tested plant extract concentrations were 0.250 mg/mL and 0.500 mg/mL. Hydroxylamine (NH_2OH) and 2.5 % *v*/v DMSO were included as positive and negative controls, respectively. The extract with revertant colonies more than twice the number of colonies in solvent control was considered mutagenic [45].

2.5. Bioassay-guided fractionation of the active extract

Since the extract of *S. lancea* exhibited the best antifungal activity, this extract was selected for purification and identification of bioactive compound(s). Sixty-four grams (64 g) of the crude ethanolic extract of *S. lancea* was partitioned with water and hexane. The water fraction was then partitioned with ethyl acetate (EtOAc) and subsequently partitioned with saturated butanol. The resulting partitions (hexane, ethyl acetate, butanol and water) were screened for their antifungal activity against the three dermatophytes and the most active partition was then subjected to silica column chromatography. A glass column was slurry-packed with silica gel 60 (particle size: < 0.063 mm; pore size: 60 Å) and eluted with 5 L of each solvent in the following sequence: hexane, hexane: EtOAc and EtOAC: methanol and increasing solvent polarity in 5 % increments to a final solvent combination of 97.5 % EtOAc: 2.5 % methanol. The pooling of the collected fractions (according to their thin layer chromatography (TLC) profiles), yielded 33 major fractions. Seven of those were selected based on quantities and tested for their antifungal activity against the selected dermatophytes as described in 2.2.2.

2.6. Liquid chromatography-mass spectrometry (LC-MS) and high-performance thin layer chromatography (HPTLC) analysis

The most active fraction was subjected to liquid chromatography-mass spectrometry (LC-MS) analysis at the Central Analytical Facility at Stellenbosch University, for untargeted chemical characterisation and identification of potential bioactive compounds. The fraction was analysed by Waters Synapt G2 in both positive and negative ionization modes at 15 voltage (v) ionization energy.

For the HPTLC analysis, glass-back, normal phase silica gel 60 F_{254} HPTLC plates (20 cm × 10 cm) were purchased from Bruno Steiner Laboratory Consultancy, Johannesburg, South Africa. All chemicals were of analytical grade (HPLC) and acquired from Merck, Johannesburg, South Africa. Experimental parameters were set according to the methods described by [46] with slight modifications. Briefly, 20 mg/mL stock concentrations of the ethanol extract of *S. lancea* (SL) and fractions (F14–15, F28–29 and F32–33) were prepared and 1 mg/mL stock concentrations of the standards were prepared in ethanol. Clean plates were visualised using the TLC Visualiser 2 (CAMAG, Switzerland). Extracts, fractions and standards were applied to the plate as bands (8 mm) using the Automatic TLC Sampler 4 (CAMAG, Switzerland) with a separation distance of 11.4 mm. The plates were then developed using the Automatic Developing Chamber 2 (CAMAG, Switzerland) using a mobile phase consisting of toluene, ethyl acetate, methanol and formic acid [8:2:0.2:0.1 (*v*/v/*v*/v)] with saturation (20 min, with saturation pad) and activation with $MgCl_2.6H_2O$ saturated solution for 10 min to obtain a constant relative humidity of 33 %. Plates were visualised under 254 nm and 366 nm in the TLC Visualiser 2. Developed plates were dried for 5 min and derivatized using a solution of acidified vanillin (2 g in 200 mL of ethanol (96 %) and 4 mL of sulphuric acid (H_2SO_4)) using the Immersion Device 3 (CAMAG, Switzerland) set at a dipping speed of 5 and dipping time of 0. Derivatized plates were then heated using the TLC Plate Heater III at 100 °C for 3 min and further evaluated using the TLC Visualizer 2 at 366 nm. A final temperature of 22 °C and 39 % relative humidity were recorded at the end of the experiment.

2.7. Antifungal activity of pure compounds

Following characterisation and identification, the compounds identified using LC-MS and HPTLC were tested for antifungal activity against the three dermatophytes according to the broth microdilution assay described in 2.2.2. The tested concentrations ranged from 0.250 to 0.02 mg/mL.

2.8. Effect of *S. lancea* extract on the morphology and ultrastructure of *Microsporum canis*

2.8.1. Treatments

Microsporum canis (ATCC 36299) was grown on PDA plates and incubated at 27 ± 2 °C for 21 days before use. Blocks of agar with new fungal growth were aseptically cut and placed on PDA plates supplemented with the ethanolic leaf extract of *S. lancea* to a final concentration of 0.312 mg/mL (2× MIC). The plates were then incubated at 27 ± 2 °C for 12, 24, 48 and 72 h. Controls included culture treated with terbinafine (positive control) (0.2 mg/mL), negative control (2.5 % *v*/v dimethylsulfoxide (DMSO)) and viability control (untreated fungus).

2.8.2. Scanning electron microscopy

Scanning electron microscopy (SEM) was performed to investigate the effect of the ethanolic leaf extract of *S. lancea* on the morphology of *M. canis* as previously described by [47], with slight modifications. Fungal agar blocks (5 × 5 mm) (control) and agar blocks of mycelium from treated plates were thinly cut with a scalpel and fixed using a fixative solution containing 2.5 % (*v*/v) glutaraldehyde and 5 % (v/v) formaldehyde in 0.075 M phosphate buffer (pH 7.0) and incubated for 12 h at 4 °C. Thereafter, the materials were washed thrice with the phosphate buffer (15 min for each wash) on an R2 rotary mixer (Pelco®, USA), and post-fixed in 1 % (*w/v*) osmium tetroxide (OsO_4) for 1 h at room temperature. The post-fixed samples were washed three times with phosphate buffer (each wash for 15 min) and dehydrated using a gradient series of ethanol solutions (30 %, 50 %, 70 %, 90 %, 95 %, and 3× 100 %) for 15 min for each series. The materials were then left in 100 % ethanol for 7 days followed by critical point drying (Julabo F12, Labortechnic GmbH, Eisenbahnstrasse, Seelbach, Germany). Dried samples were mounted onto aluminium stubs lined with conductive carbon adhesive tape and coated with carbon using a sputter coater (Q150TES, Quorum Technologies LTD, England) and observed under a SEM (Ultra plus, Zeiss, Oberkochem, Germany) operating at 2.0 kV. Images were taken at 5000× magnification.

2.8.3. Transmission electron microscopy

Transmission electron microscopy (TEM) was performed to observe the effect of the ethanolic leaf extract of *S. lancea* on the ultrastructure of *M. canis* as previously described by [48], with modifications. The samples were prepared in the same manner as the SEM samples (Section 2.8.2) with the exception that the last dehydration step (100 % ethanol) was for 1 h. Following dehydration, fungal cells were placed in a mixture of epoxy resin (Agar 100) and 100 % ethanol (1:1) overnight on an R2 rotary mixer and embedded in epoxy resin the following day. The embedded samples were placed in an oven (Series 2000, Scientific) at 60 °C for 24 h for polymerization. Thin sections of the specimens were cut using an ultramicrotome (Reichert, Ultracut S) and mounted onto carbon-coated copper grids.

To enhance contrast, the specimens were stained on dental wax using filtered solutions (0.22 μm filter paper) of saturated aqueous uranyl acetate and 4 % lead citrate. The grid sections were then evaluated using a JEM-1400 Flash TEM (JEOL, Japan).

2.9. Effect of *S. lancea* extracts on the virulence of *M. canis*

2.9.1. Infection of human hair with *M. canis*

Human hair was obtained from a healthy 30-year-old female. Hair strands were washed with detergent, thoroughly rinsed with distilled water and left to dry in an oven at 35–40 °C overnight. Hair strands were then defatted by soaking 20 g of hair in 200 mL of chloroform: methanol (1:1) for 1 h at room temperature followed by rinsing with distilled water and overnight drying at 60 °C in an oven (310-drier, Labotec, South Africa) [49]. Hair strands were then cut into 1–2 cm segments for subsequent co-culture with *M. canis*.

Microsporum canis (ATCC 36299) was grown on PDA for 21 days at 27 ± 2 °C. One-hundred milligrams (100 mg) of the pre-treated hair was inoculated with *M. canis* conidial suspension (5×10^6 CFU/mL) in 18 mL of RPMI 1640 medium supplemented with 0.46 g/L KH_2PO_4, 0.5 g/L $MgSO_4$, 1.0 g/L K_2HPO_4 (minimal medium), pH 5.8, in a 50 mL Erlenmeyer flask, [50]. The fungal-hair cultures were incubated on a shaking incubator for 1, 2 and 6 days at 27 ± 2 °C to allow expression of virulent genes prior to treatment with 2 mL of ethanolic leaf extract of *S. lancea* (0.312 mg/mL). The treated cultures were incubated for a further 24 h. Cultures of fungal conidia grown in the absence of hair, and fungi grown in a medium supplemented with hair and no extract were also prepared.

At the end of each incubation period (2, 3 and 7 days), fungi had clustered around the hair and the mycelia were removed with sterilized forceps and rinsed thrice with distilled water and blot-dried on a sterile Whatman no.3 filter paper. Approximately 100 mg of the washed fungi was lyophilized and triturated in liquid nitrogen and stored at -80 °C overnight.

2.9.2. RNA extraction, cDNA synthesis and RT-qPCR

Total ribonucleic acid (RNA) of *M. canis* was extracted using the RNeasy Plant Mini Kit (Qiagen, South Africa, Johannesburg) according to the manufacturer's instructions followed by DNase I (ThermoFisher Scientific, South Africa) treatment to eliminate potential genomic deoxyribonucleic acid (DNA) contamination. The NanoDrop™ 2000/2000c spectrophotometer (ThermoFischer Scientific, USA) was used to assess the quantity and purity of the extracted RNA by assessing absorbance at 230 nm, 260 nm and 280 nm wavelength. A 2 % (*w/v*) denaturing agarose gel electrophoresis was used to assess the RNA integrity, separated at 85 voltage for 40 min.

A RevertAid H-minus First Strand cDNA synthesis kit (ThermoFisher, South Africa) was used to synthesize the complementary DNA (cDNA) with random hexamer primer by reverse transcription of 2 μg RNA following the manufacturer's instructions. Two separate quantitative Reverse Transcription Polymerase Chain Reaction (RT-qPCR) reactions amplifying the target gene, *SSU1*, responsible for the activation of keratin degradation, and β-actin, a housekeeping gene, were prepared with primer sets indicated in Table 1. Each 10 μL reaction mixture contained 5 μL of Luna Universal qPCR Master Mix (New England Biolabs, Germany), 0.25 μL of each primer (100 μM), 1 μL of cDNA (0.1 μg/μL) and 3.5 μL of nuclease-free water.

For standard curve determination, cDNA samples from all time-points were pooled together and diluted 1:10 and 1 μL was used in a 10 μL RT-qPCR reaction using the gene-specific primers amplifying the *SSU1* and β-act genes (Table 1). The RT-qPCR reactions were performed on a C1000 thermal cycler (Bio-Rad, California) with an initial denaturing step at 95 °C for 10 min, followed by 35 cycles at 95 °C for 15 s, 60.5 °C for 15 s and 60 °C for 20 s (Table 2). All RT-qPCR experiments were performed on three technical and three biological replicates. The melt curve peaks, and standard curve taken from the CFX maestro 2.2 software were used to determine the specificity and efficiency of the amplification. Gene expression levels were quantified by the comparative Ct method ($2^{\sim\Delta\Delta Ct}$) using β-act as the normaliser gene as described by [53]. Quantification of the expression of the *SSU1* gene relative to β-act was done in two parts. The first quantification was done to confirm the success of hair infection, the untreated fungus (F) samples were compared to fungus grown in media supplemented with hair but without extract (FH). The second part was the quantification of *SSU1* expression in stimulated samples treated with *S. lancea* leaf extract (FHE); FHE-samples were analysed in comparison to FH-samples.

2.9.3. Statistical analysis

The Log ($2^{-\Delta\Delta Ct}$) values of real-time qPCR were compared by two-tailed *t*-tests using GraphPad Prism 4.0 comparing the samples at each incubation period. A *p*-value <0.05 was considered statistically significant. The data is expressed as mean ± standard deviation (SD) of three independent experiments (n = 3).

Table 1
Details of the specific primers used for qPCR.

Gene symbol/ accession no.	Gene name	Primers, 5′ - 3′ forward 3′-5′ reverse	Synthesis	Reference
SSU1 MCYG_08415	Sulfite-efflux pump (target)	AAGAGCTTC AGGTCACAGCC AAGCCCGGGAAACTGGTATG	IDT, South Africa	[51]
β-act XM_002845542 HKG	β-actin (housekeeping)	CTCCTGAGGCTCTCTTCC GTAGTACCGCCGGACATG	Inqaba Biotec, South Africa	[52]

Table 2
The qPCR thermal profile of *SSU1* and β-act genes.

Phase	Time	Cycles	Temperature (°C)
Initial denaturation	1 min	1	95
Denaturation	15 s	35	95
Annealing	15 s		60.5
Extension	20 s		60
Melting temp	10 s	1	95
	15 s		60.5
	10 s		95

3. Results

3.1. Anti-dermatophytic screening

3.1.1. Antifungal activity of crude extracts

The highest extraction yield was observed for *Searsia lancea* (19.44 % w/w) compared to *M. comosus* (15 % *w*/w) and *W. salutaris* (10.34 % w/w). All three extracts showed antifungal activity against the tested dermatophytes (Table 3). *Melianthus comosus* showed noteworthy activity against all three pathogens exhibiting a minimum inhibitory concentration (MIC) of 0.470 mg/mL. A notable activity of *W. salutaris* was demonstrated against *M. gypseum* and *T. mentagrophytes* (0.940 mg/mL), however, the extract exhibited the lowest activity against *M. canis* (7.500 mg/mL). *Searsia lancea* exhibited the highest activity (lowest MIC) against *M. canis* (0.156 mg/mL) while an MIC of 0.313 mg/mL was recorded against *M. gypseum* and *T. mentagrophytes.* Griseofulvin showed a MIC value of 0.080 mg/mL against *M. gypseum* and no inhibitory effects on *M. canis* and *T. mentagrophytes* which only showed reduced growth when compared to the fungal growth control.

3.1.2. Interactive combination studies

All obtained CI values indicated synergistic interaction between the plant extracts except for the 7:3 ratio of *S. lancea*: *M. comosus* against *T. mentagrophytes*, which showed to be antagonistic (CI value of 4.83). Table 4 shows the extract ratios which exhibited synergistic interaction (CI value <0.5). The best antifungal activity was observed against *M. canis,* with increasing concentration of *W. salutaris* in relation to *S. lancea* with the best synergy observed at a ratio of 9:1. The best synergistic interaction against *T. mentagrophytes* was observed with the combination of *M. comosus: W. salutaris*, 2:8 ratio.

3.2. In vitro antiproliferative activity

Melianthus comosus and *W. salutaris* ethanol extracts were non-toxic to the HaCaT cells IC_{50} values of 111.40 ± 2.05 μg/mL and 191.55 ± 1.20 μg/mL, respectively (Table 5). The *S. lancea* leaf extract had an IC_{50} value of 82.28 ± 4.99 μg/mL indicating moderate toxicity (Table 3). The combination of *S. lancea* and *M. comosus* (9:1) was found to inhibit 50 % of cell proliferation at 131.42 ± 42.14 μg/mL. The concentration of each component at this ratio was 117.9 μg/mL of *S. lancea* and 13.10 μg/mL of *M. comosus*. All extracts were statistically different to the positive toxic inducer, Actinomycin D, indicating that they were not as toxic as the positive control, Fig. 1.

3.3. Mutagenicity test

All extracts and tested concentrations were found to be non-mutagenic except *W. salutaris* which showed mutagenic potential at the highest tested concentration of 500 μg/mL. The number of reverted colonies for *W. salutaris* (500 μg/mL) was 18 colonies compared to 34 of the positive control. Three colonies were reverted in the *M. comosus* (500 μg/mL), which is considered non-mutagenic as they were equal to the number of revert colonies in the negative control. All other leaf extracts showed no ability to revert colonies of *E. coli*. Table 5 shows the number of reverted colonies for each sample.

3.4. Bioassay-guided fractionation

The partitioning of the ethanolic leaf extract of *S. lancea* with various solvents (hexane, EtOAc, butanol and water) yielded five partitions, namely the: hexane fraction (10.5 g), EtOAc fraction (22.6 g), butanol fraction (10.4 g), water fraction (11.1 g) and interphase layer fraction (5.8 g) between butanol and water. The interphase partition did not dissolve in any organic solvent, as such, it was not tested against the dermatophytes. Of the four tested partitions, hexane and EtOAc showed comparable MICs against *M. gypseum* (0.156 mg/mL) and *T. mentagrophytes* (0.313 mg/mL). The lowest MIC (highest activity) was recorded for EtOAc against *M. canis* (0.078 mg/mL). The EtOAc fraction showed noteworthy activity (below 1 mg/mL) [20] against all three dermatophytes.

Of the seven tested fractions, fraction 28 (F28) (1.62 g) showed no activity against all three dermatophytes. The best activity was observed with fraction 15 (F15) (0.460 g), with MIC of 0.033 mg/mL and 0.063 mg/mL recorded against *M. canis* and *T. mentagrophytes*, respectively (Table 3).

3.5. Liquid chromatography-mass spectrometry and high-performance thin layer chromatography (HPTLC)

The LC-MS predicted the presence of gentisic acid or protocatechuic acid as one of the major constituents of the F15 fraction. The retention time (R_t) of this compound was 5.499 min with $C_7H_5O_4$ as the chemical formula. The spectrum showed a deprotonated fragment ion of 153 *m/z* (Fig. 2A). The LC-MS spectrum also presented a peak showing 285 m/z (Fig. 2B) with Rt of 7.272 min and a chemical formula of $C_{16}H_{13}O_5$ which was tentatively identified as sakuranetin.

The HPTLC profiles of the ethanolic extract of *S. lancea,* the active fractions collected from the purification of the ethyl acetate fraction (F14–15, 28–29, 32–33), compounds predicted by LC-MS (sakuranetin, gentisic acid, protocatechuic acid) and compounds previously isolated from the *S. lancea* (eicosane, lignoceryl alcohol; nonadecanol, and pentadecanol), were obtained. The developed HPTLC plate observed under UV at 254 nm indicated the presence of protocatechuic acid with a retention factor (R_f) value of 0.12 (indicated in red) (Fig. 3A). The HPTLC profile of the ethanolic extract (SL) with a prominent band at R_f 0.13, fraction 28 (R_f 0.13) and 29 (R_f 0.14). However, in the HPTLC plates derivatized using vanillin the absence of the deep orange colour in the "PCA" track suggests that the band present in the extract and fractions was likely not protocatechuic acid (Fig. 3C-E).

The HPTLC plates illuminated under UV254 nm showed the R_f value of sakuranetin at 0.49. The presence of sakuranetin was also present in

Table 3
The antifungal activity of the crude extracts, partitions, fractions and pure compounds.

Dermatophytes	Minimum inhibitory concentration (mg/mL)															
	Melianthus comosus	*Warburgia salutaris*	*Searsia lancea*	Positive control	Hexane	EtOAc	Butanol	Water	F14	F15	F16	F28	F29	F32	F33	Gentisic acid	Sakuranetin
Microsporum canis ATCC 36299	0.470	7.500	0.156	–[a]	0.156	0.078	1.25	2.5	0.125	0.033	0.125	0.125	–[a]	0.500	0.125	0.250	0.250
Microsporum gypseum (clinical isolate)	0.470	0.940	0.313	0.08	0.156	0.156	2.5	–[a]	–[a]	0.125	0.500	0.250	–[a]	–[a]	0.125	0.125	0.063
Trichophyton mentagrophytes ATCC 9355	0.470	0.940	0.313	–[a]	0.313	0.313	–[a]	–[a]	0.500	0.063	0.250	0.250	–[a]	–[a]	0.125	–[a]	0.004

EtOAc: ethyl acetate; F14: fraction 14; F15: fraction 15; F16: fraction 16; F28: fraction 28; F29: fraction 29; F32: fraction 32; F33: fraction 33. Positive control = Griseofulvin.
[a] No activity observed at the highest tested concentration.

fractions F32 and F33 with R_f values of 0.47 (indicated in blue) (Fig. 3A). The HPTLC plate derivatized with vanillin showed sakuranetin as a red-orange band which was present in F32 and F33 but also showed the presence of a much lighter red band at R_f 0.51 present in F15 (Fig. 3C-E). Confirmation of the presence of sakuranetin in fractions F32 and F33 can be observed in the HPTLC plate derivatized with vanillin and illuminated under UV366 nm which showed a dark hue surrounded by a blue halo at R_f 0.49 (in the track labelled SKN) and the tracks with fractions F32 and F33 at an R_f of 0.47 (indicated in yellow). Interestingly, the pink band in fraction F15 also showed a dark hue at an R_f of 0.51 indicated by the yellow arrow, suggesting that this could be sakuranetin (Fig. 4) and therefore supports the LC-MS analysis. Previously isolated compounds could not be visualised by HPTLC using the selected derivitization methods and may require a more sensitive technique to detect the low concentrations that may be present in the fractions. Comparison of the fractions and the compound sakuranetin in derivatized HPTLC plates using vanillin are given in Fig. 5A-C.

3.6. *Antifungal activity of identified compounds*

Both gentisic acid and sakuranetin exhibited antifungal activity against the tested dermatophytes, in differing degrees. *Trichophyton mentagrophytes* was the most susceptible pathogen to sakuranetin which had an MIC of 0.004 mg/mL compared to 0.250 mg/mL (*M. canis*) and 0.063 mg/mL (*M. gypseum*) (Table 1). Gentisic acid exhibited no activity against *T. mentagrophytes* at the highest tested concentration of 0.250 mg/mL, however, MICs of 0.250 and 0.125 mg/mL were recorded for *M. canis* and *M. gypseum*, respectively.

3.7. *Effect of S. lancea extracts on morphology and ultrastructure of Microsporum canis*

3.7.1. *Scanning electron microscopy*

The SEM micrographs of the untreated and DMSO-treated *M. canis* mycelium showed no differences in their structure while alterations were observed in those treated with terbinafine and leaf extract. On the surface, in the untreated control and the solvent control (2.5 % DMSO), the hyphae appeared to be unaltered, showing uniform and tubular hyphae. At the 12 and 24- h time-points, the samples of all four treatments showed cylindrical, roughened fungal structures with signs of new growth (Fig. 6A-H). At 48 h, the negative control samples had young hyphae, which were smooth, uniform, and tubular (Fig. 6I and J). In contrast, the terbinafine-treated fungus remained rough and flattened. The young hyphae which were observed at 12 and 24 h, appeared to have collapsed, thereby abolishing the development of new hyphae and establishment of mycelium (Fig. 6K and O). The same was noticed for the fungal sample treated with *S. lancea* extract (48 and 72 h) which appeared damaged as the hyphae began to appear porous (Figs. 6L, 7A-i, ii, and Biv). At 72 h, the fungus treated with *S. lancea* showed signs of breakage of hyphae into fragments of about 2.5 μm observed (Fig. 7B-iii).

3.7.2. *Transmission electron microscopy*

The TEM micrographs of the untreated *M. canis* control showed a homogenous cell matrix with structural integrity with the nucleus, mitochondria, intact inner and outer cell walls, and cell membrane being observed at all the incubation time-points (Fig. 8A, E, I, & M). Similarly, the DMSO-treated *M. canis* exhibited a regular cell wall structure, a uniform and unfolded plasma membrane with a homogenous matrix (Fig. 8B, F, J, & N). In contrast, the hyphae exposed to terbinafine (positive control), showed accumulation of lipid droplets at 12 h (Fig. 8C) and 24 h of incubation (Fig. 8G) and the whole intracellular matrix was filled with electron-dense material at 48 h (Fig. 4.3K) and 72 h (Fig. 8O). The *M. canis* hyphae treated with the ethanolic extract of *S. lancea* showed no apparent organelles, and shrinkage of the plasma membrane was observed 12 h post-treatment (Fig. 8D). At 24 h of

Table 4
Synergistic interaction of the crude extracts of *M. comosus, S. lancea* and *W. salutaris* against dermatophytes.

Dermatophytes	Griseofulvin	Plant combination	[a]MIC (μg/mL)		Ratio	Combination index
			Individual	Combination		
Microsporum canis ATCC 36,299	*n/a*	*Sl* + *Mc*	156 + 470	14.063: 32.813	3:7	0.160107
		Sl + *Ws*	156 + 7500	09.375: 84.375	1:9	0.051229
		Mc + *Ws*	470 + 7500	28.125: 18.750	6:4	0.062468
Microsporum gypseum (clinical isolate)	*0.08*	*Sl* + *Mc*	313 + 470	09.375: 84.375	1:9	0.199893
		Sl + *Ws*	313 + 940	18.750: 04.688	8:2	0.044981
		Mc + *Ws*	470 + 940	21.094: 02.244	9:1	0.047477
Trichophyton mentagrophytes ATCC 9533	*n/a*	*Sl* + *Mc*	313 + 470	42.188: 04.688	9:1	0.099947
			313 + 470	28.125: 18.750	6:4	0.099947
		Sl + *Ws*	313 + 940	21.094: 02.344	9:1	0.047477
		Mc + *Ws*	470 + 940	09.375: 37.500	2:8	0.060011

MIC: minimum inhibitory concentration; Sl: *Searsia lancea*; Mc: *Melianthus comosus*; Ws: *Warburgia salutaris*; n/a: no observed MIC.

Table 5
Cytotoxicity and mutagenicity of plants traditionally used in treatment of skin infections.

Sample	Cytotoxicity	Mutagenicity	
	IC_{50}[a] ± SD[b] (μg/mL)	RC[c] (250 μg/mL)	RC[d] (500 μg/mL)
Searsia lancea	82.28 ± 4.99	0	0
Melianthus comosus	111.40 ± 2.05	0	3
Warburgia salutaris	191.55 ± 1.20	0	18
SL[e]:MC[f] (9:1)	131.20 ± 42.14	–	
2.5 % v/v DMSO		3	
Positive control[g]	0.004 ± 0.003[g]	34[h]	

[a] Fifty percent inhibitory concentration.
[b] Standard deviation.
[c] Revert colonies at 250 μg/mL of the plant extract.
[d] Revert colonies at 500 μg/mL of plant extract.
[e] *Searsia lancea*.
[f] Melianthus comosus.
[g] Positive control for cytotoxicity (Actinomycin D).
[h] Positive control for mutagenicity (Hydroxylamine.

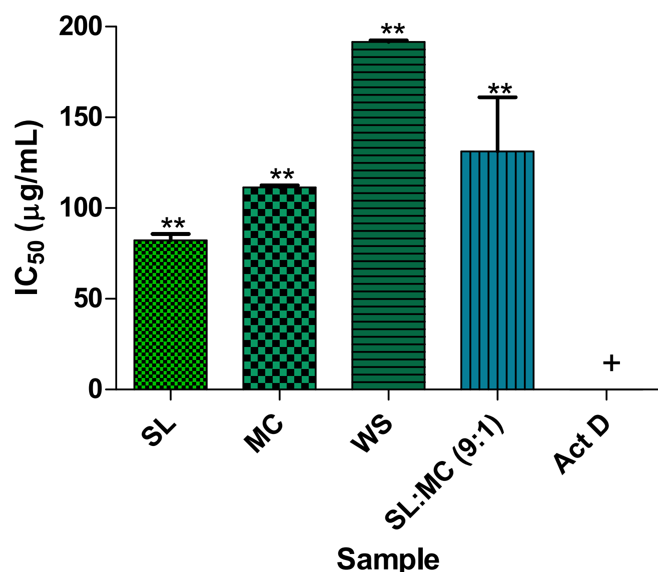

Fig. 1. The IC_{50} of plant extracts against HaCat cells. Data is shown as mean ± SD (n = 3). One-way ANOVA followed by Dunnett's multiple comparison test was used to calculate significance where ** $p < 0.01$ indicated statistical significance compared to the control (+). SL = *Seasia lancea*; MC = *Melianthus comosus*; WS = *Warburgia salutaris*; SL:MC = *S. lancea: M. comosus* and Act D = Actinomycin D.

treatment, the extract-treated fungus showed abnormal distribution of polysaccharides in the cytoplasm (Fig. 8H). In addition, the hyphae exhibited folding of the plasma membrane, as well as plasmolysis, (Fig. 9A). Moreover, at 48 h, the cell wall disintegrated and electron-dense material was also observed (Fig. 8L) and extensive disruption of the cytoplasm (Fig. 9B). Fig. 8P shows total degradation of the plasma membrane and apparent extravasation of the intracellular material of the fungus treated with the extract for 72 h.

3.8. Effect of *S. lancea* extracts on the virulence of *M. canis*

3.8.1. RNA extraction, cDNA synthesis and RT-qPCR

Experiments were performed on RNA samples with absorbance A260/A280 ratios between 1.9 and 2.1 and A260/A230 ratios higher than 2.0. The agarose gel electrophoresis showed that the extracted RNA was intact.

The melt peak analysis demonstrated a single homogeneous peak for primers for both genes indicating specific gene amplification (Fig. 10A and B). The calculated PCR efficiencies for the *SSU1* and β-act genes were 96.6 and 109 %, respectively (Fig. 10C and D). The efficiency curve of the two genes showed a linear correlation coefficient (R^2) of 0.949 for the *SSU1* gene and 0.990 for β-actin. The raw cycle threshold (number of cycles required to replicate enough RNA to be detected, Ct) values were used to calculate the average Ct for each amplicon in each replicate. The samples exhibited Ct values ranging from 18.45 to 31.06.

The induction of the *SSU1* gene was observed in each incubation period. The expression of the *SSU1* gene in hair-supplemented samples was significantly higher than that of the control (fungus only) ($p < 0.05$) at 2 days (FH-2D) and 3 days (FH-3D) (Fig. 11A). The most significant induction was noted at the 3-day incubation period (FH-3D) ($p < 0.01$) showing an 8-fold increase in gene expression relative to 2-days of growth. A high fold change was observed in the 7-day fungi-hair complex (FH-7D) (13-fold), however, this was not significantly different ($p > 0.05$) from that of conidial control (fungus only) for the same time-point.

The assessment of *SSU1* gene expression in extract-treated fungi showed reduced expression in treated samples relative to the untreated samples. Fig. 11B shows the results of the change in expression levels of the co-culture after exposure to the extract. At 2-days (FHE—2D) and 7-days (FHE—7D) incubation, the expression of the *SSU1* gene was significantly decreased, exhibiting a 0.34 and 0.8-fold reduction, respectively. The evaluation of expression at 3-days (FHE-72) reported a 3.5-fold decrease which was not significantly different from that of the control of the same period.

4. Discussion

The selected plant extracts *M. comosus*, *W. salutaris* and *S. lancea* which showed noteworthy activity against all three dermatophytes, with *S. lancea* exhibiting the best activity against *M. canis*. According to Van

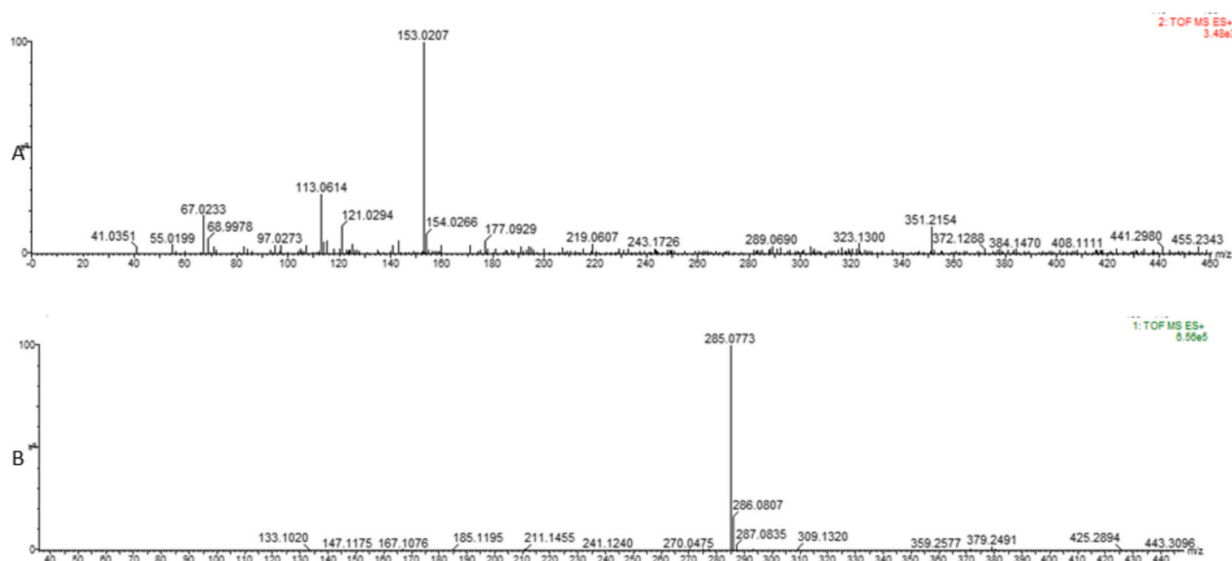

Fig. 2. The LC-MS spectrum of A) gentisic acid and B) sakuranetin showing the *m/z* peak of the two compounds.

Fig. 3. A) Developed HPTLC plate illuminated under UV254 nm, B) under UV366 nm, C) HPTLC plate derivatized using vanillin illuminated under visible light from the top, D) visible light from the top and bottom, E) visible light from the top and F) HPTLC plate derivatised with vanillin and illuminated under UV366 nm. SL = *Searsia lancea* ethanol extract; F14, F15, F28, F29, F32 and F33 = fractions 14, 15, 28, 29, 32, 33; IEC = eicosane; GA = gentisic acid; LIG = lignoceryl alcohol; NON = nonadecanol; PCA = protocatechuic acid; PEN = pentadecanol; SKN = sakuranetin.

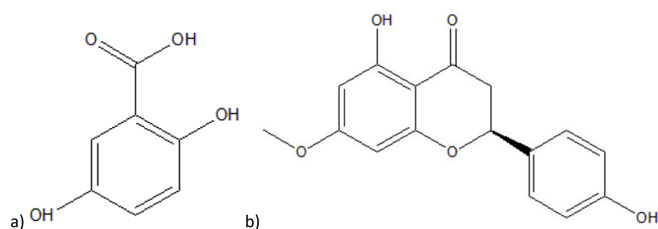

Fig. 4. Chemical constituents of fraction 15 A) Gentisic acid and B) Sakuranetin.

Vuuren [20] noteworthy antifungal activity is defined as an MIC <1.0 mg/mL. The extracts were tested in combination, attempting to improve their antifungal activity and reduce toxicity. Several studies have investigated the combination of two or more extracts for improved antifungal activity [54,55]. Most of the tested plant extract combinations showed synergistic effects against the tested dermatophytes. More pronounced synergism was observed where the concentrations of *W. salutaris* were high in relation to the other two leaf extracts. However, when the extracts were tested individually, *S. lancea* exhibited better antifungal activity than *W. salutaris.* No information could be found on the synergistic studies or traditional usage of *W. salutaris* with the two plants in this study.

Searsia lancea was found to be moderately toxic to HaCaT. Although *S. lancea* showed some antiproliferative effects, its combination with *M. comosus* (9:1) showed to be non-cytotoxic. This ratio was evaluated following the results of the individual extracts and was selected in order to reduce the cytotoxicity of *S. lancea* on HaCat cells, and it allows the use of a higher concentration of *S. lancea* without exposing the skin cells to the moderate toxic effects of *S. lancea* because the *M. comosus* extract appears to reduce the toxicity of *S. lancea.*

Although *S. lancea* showed moderate toxic effects on the human skin cells, it exhibited no mutagenic effects on the DNA of *E. coli* at the highest tested concentration of 500 μg/mL. The current findings of *S. lancea* extract correlate with those of Mulaudzi et al. [56] where an 80 % ethanol *S. lancea* ethanolic leaf extract was found to be non-mutagenic to the *Salmonella typhimurium* TA98, TA100 and TA102 isolates. Due to its low MIC against *M. canis,* low cytotoxic and no mutagenic effects, *S. lancea* was subjected to subsequent investigations.

The EtOAc fraction of *S. lancea* showed noteworthy activity against all three dermatophytes and exhibited a two-fold increase in activity against *M. canis* and *M. gypseum*. Subsequently, the EtOAc partition was subjected to silica column chromatography for further purification. Of the seven fractions evaluated for antifungal activity, the best activity was observed with fraction 15, which exhibited a four-fold increased activity against *M. canis* and *T. mentagrophytes*. This fraction was subsequently characterized by LC-MS and HPTLC. Deprotonated fragment ion at *m/z* 153 of the spectrum led to the conclusion that fraction contains gentisic acid instead of protocatechuic acid [57,58]. The HPTLC confirmed the presence of sakuranetin in F15 supporting the identification of sakuranetin. The LC-MS also showed fragmentation ion at m/z 285 which correlates with the findings by Afifi et al. [59] who reported that sakuranetin derivatives show a peak at m/z 285.

Sakuranetin is a phytoalexin belonging to the group of methoxylated flavanones and has been isolated from a wide range of plants [60,61]. No literature could be found on the isolation of this compound from *S. lancea*. It has been reported to have antifungal properties against a wide variety of fungal pathogens including *Candida* species and some dermatophytes [61–63]. The current study showed *T. mentagrophytes* to be the most susceptible pathogen to sakuranetin showing a MIC of 0.004 mg/mL compared to 0.250 mg/mL (*M. canis*) and 0. 063 mg/mL (*M. gypseum*). This correlates with the study by Pacciaroni et al. [64] which also found *T. mentagrophytes* to be the most sensitive against sakuranetin. Gentisic acid is a diphenolic compound which is a derivative of benzoic acid and a metabolite of aspirin [65]. No information could be found on the activity of this compound against dermatophytes.

Although there are numerous reports disclosing *in vitro* antifungal efficacy of medicinal plants, there is a limited number of studies that determine their MoA. Determining the MoA may be crucial in overcoming regulatory challenges involved in drug approval for clinical use and increasing the number of plant-derived alternatives to conventional anti-fungal agents. The MoA of *S. lancea* leaf extract was investigated by assessing its effect on the structure of *M. canis* (hyphal surface structure and internal organelle structure) following treatment with the extract. The changes observed on SEM micrographs were flattening, compression and clustering of the hyphae in the *S. lancea*-treated samples. The smooth and tubular hyphae were observed in the negative controls (untreated and DMSO-treated) which symbolised new growth [66]. However, the hyphae of *M. canis* treated with terbinafine and the leaf extract remained rough, this is a typical characteristic of the macroconidia of *M. canis* [66]. Furthermore, the mycelium appeared to be dried and wrinkled, which is caused by the extravasation of the cell cytoplasm [47]. This dried mycelium further suggests the accumulation of the lipophilic components of the extract on the membranes and subsequent energy depletion [47]. Evidence of extravasation was further provided by the TEM analysis, which showed cytoplasm contents scattered and unenclosed within the plasma membrane, in the 12–24 h samples. Notably, other studies reported similar observations regarding the hyphae of *M. canis* and other dermatophytes, using SEM and TEM, although they treated the hyphae with different plant extracts [26,47,67–69].

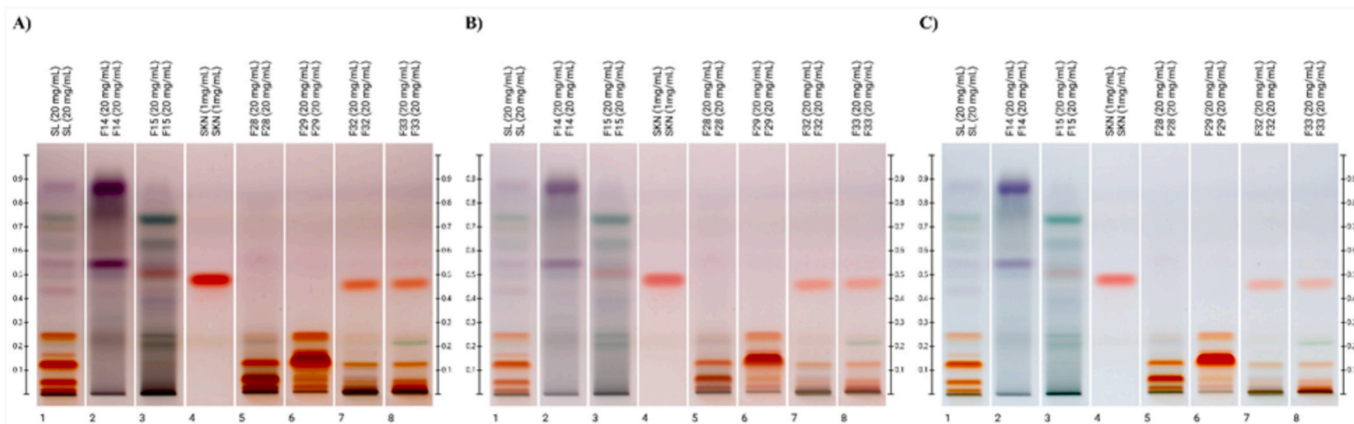

Fig. 5. HPTLC plates derivatized with vanillin, illuminated with visible light from A) the bottom, B) the top and bottom and C) the top showing the presence of sakuranetin.

Fig. 6. Scanning electron micrographs of *Microsporum canis* ATCC 36299 at four-time points, showing new growth (white arrows), smooth cylindrical hyphae (blue arrow) and compact mycelium (C) viewed at 5000× magnification. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

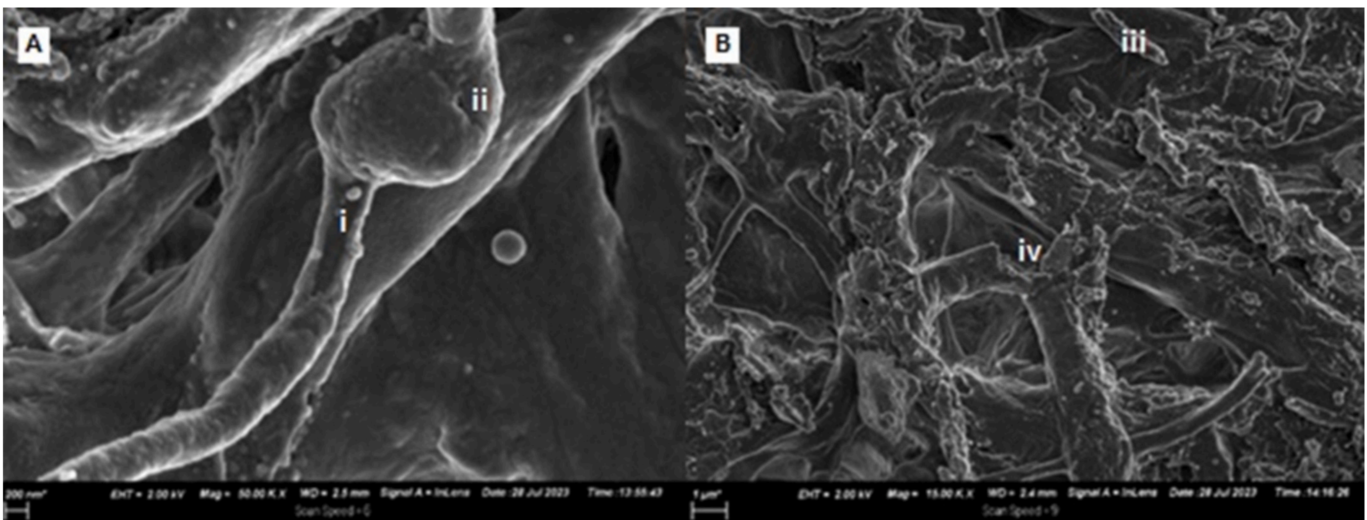

Fig. 7. The hypha of *Microsporum canis* treated with *Searsia lancea* leaf extract (0.312 mg/mL) after 72 h showing breakage (i-ii, iv) and fragmentation (iii) at 50000× magnification (A) and 15,000× magnification (B).

Moreover, the TEM analysis showed a loss of integrity of the plasma membrane in samples treated with the *S. lancea* ethanolic leaf extract for 48–72 h. In a previous study, gas chromatography–mass spectrometry showed the ethanolic extract to have 2-Propen-1-amine, N, N-bis (1-methylethyl), which is a derivative of 2-Propen-1-amine [70]. This compound belongs to the class of antifungals called allylamines and they

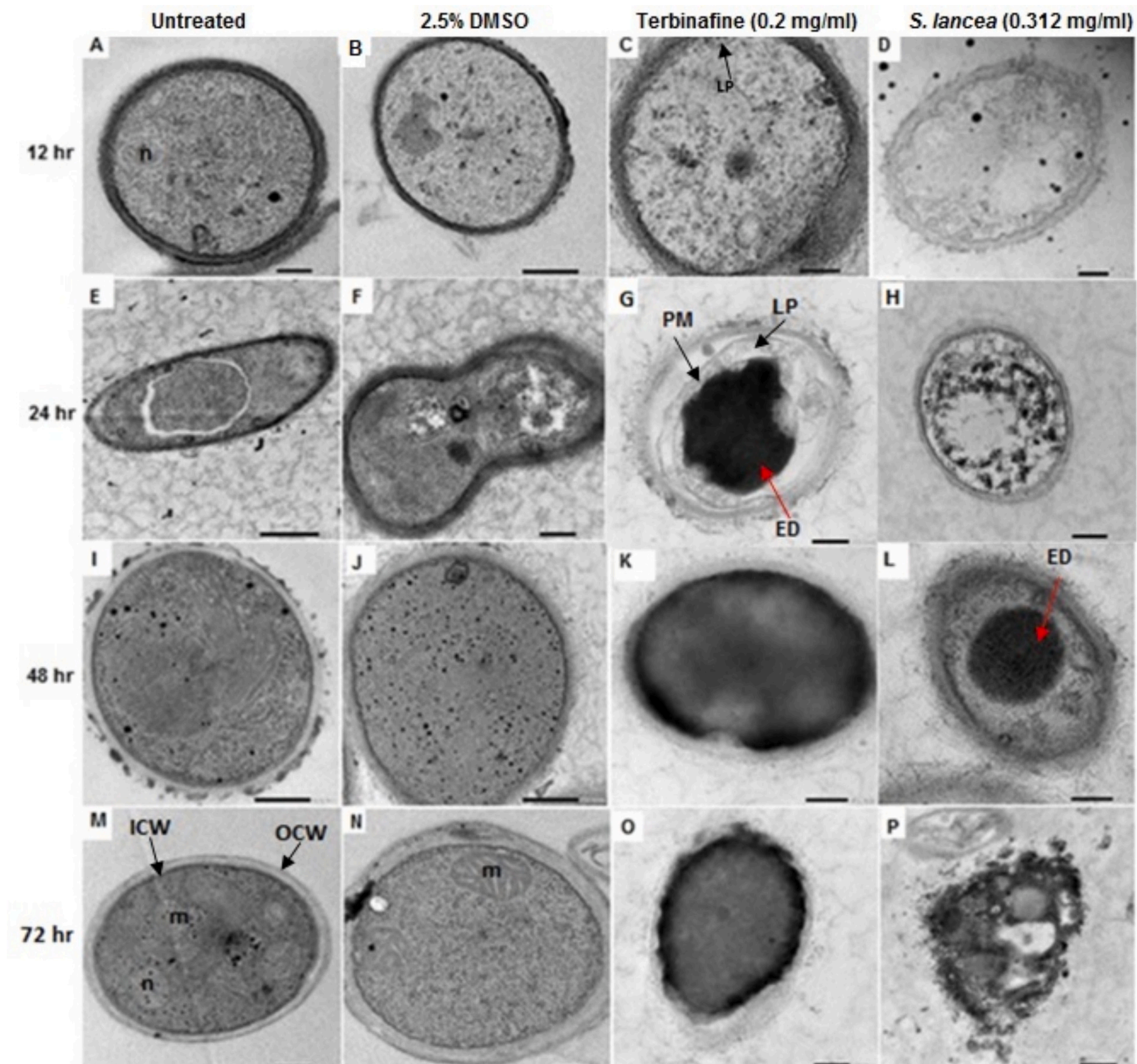

Fig. 8. Transmission electron micrographs of *Microsporum canis* ATCC 36299 at four time-points. n = nucleus; m = mitochondrion; OCW = outer cell wall; ICW = inner cell wall; ED = electron dense material; LP = lipid droplets and PM = plasma membrane. Scale bars: A, D, G, K-L, N-O: 200 nm; C, *E*-F, H-J, M & P: 500 nm; B: 1.0 μm.

inhibit fungal growth by blocking the conversion of squalene into 2, 3-squalene, thereby abolishing the synthesis of ergosterol [71]. The accumulation of squalene causes disorganisation of the cytoplasm [71–73]. The accumulation of squalene could explain the disruption of the cytoplasm observed in leaf extract-treated *M. canis* from 24 h while the inability to synthesize ergosterol explains the extravasation at 72 h [73,74].

During infection, dermatophytes secrete proteases which degrade the keratin protein into oligopeptides and free amino acids, which serve as a source of nutrients for the fungi [51,75]. The degradation of hard keratinized structures (hair, skin and nails) is possible after the degradation of disulfide bridges (S—S) following the excretion of sulfite (SO_3^{2-}), which acts as a reducing agent. The sulfite is excreted by the fungal sulfite efflux pump which is encoded by the *SSU1* gene. Reviews by McCarthy et al. [76] and Ivanov et al. [77] suggested that gene expression of this cellular efflux pump could serve as a potential target for novel antifungal drugs.

This study showed the upregulation of *SSU1* in *M. canis* grown in a medium supplemented with hair. Although a high fold change was observed in the 7-day fungi-hair complex, it was not significantly different compared to the conidial control of the same time-point. This could be because the invasion of the keratinous structure is reported to be completed on day 7 following adherence of fungi to the keratinous substrate [78,79]. The most significant upregulation was observed at 3-days of incubation ($p < 0.01$). This correlates with the observation by Ciesielska et al. [51], who reported that the degradation of keratin releases amino acids within 2–3 days. According to Ciesielska et al. [51], the *SSU1* gene is strongly upregulated by high concentrations of cysteine following the initial cleavage of the disulfide bridges. The concentration of cysteine is high during the release of amino acids from keratin which occurs between 48 and 96 h and confirms successful degradation of hair keratin [79]. The *SSU1* gene expression was significantly inhibited after 2 and 7 days of exposure to *S. lancea* ethanolic leaf extract. However, the expression of *SSU1* after 3 days showed no significant difference to that of the control (FH-3D). The observed overall trend shows inhibition of *SSU1* gene expression in treated samples. However, at this time-point (3 days), it is also where the expression was repressed the most, recording a 3.5-fold decrease.

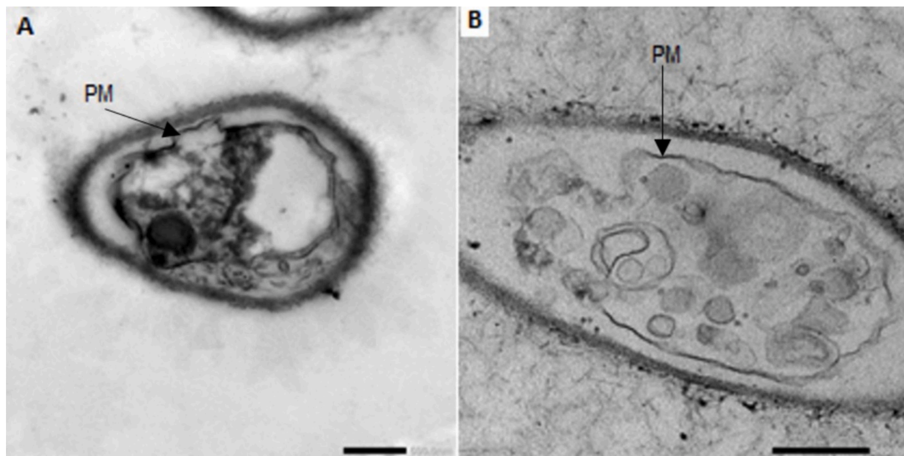

Fig. 9. The disruption of the plasma membrane of *M. canis* treated with 0.312 mg/mL ethanolic leaf extract of *S. lancea* for A) 24 h and B) 48 h. PM = plasma membrane. Scale bar: 500 nm.

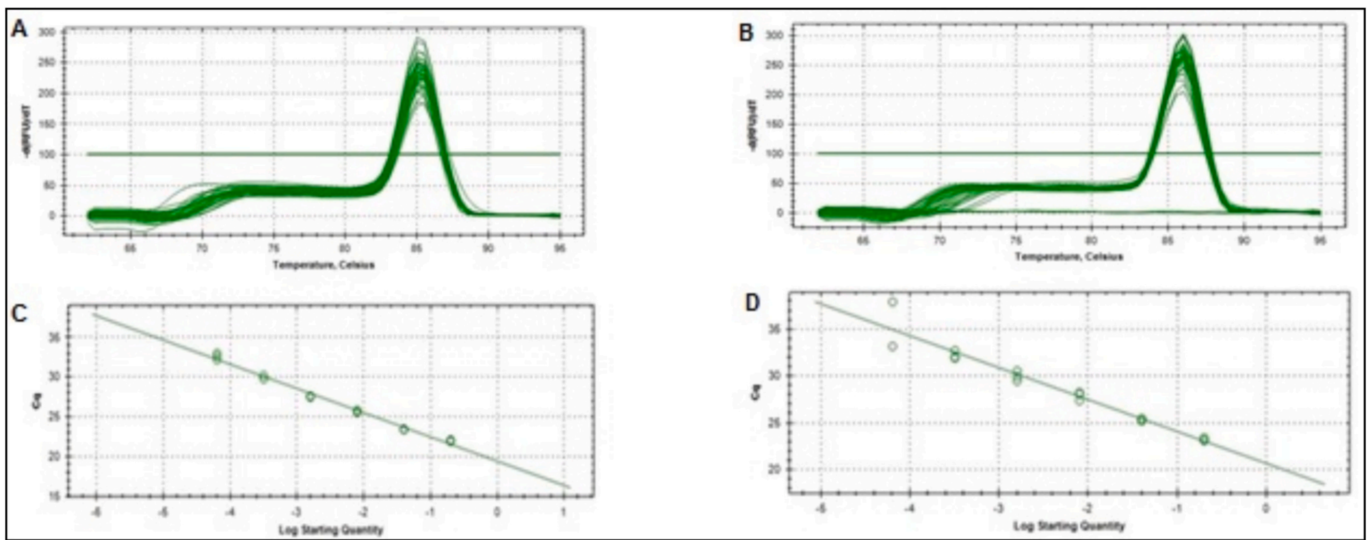

Fig. 10. The melting curve of β-act (A) and SSU1 (B) showing a single peak and the standard curve of β-act (C) and SSU1 (D) showing the qPCR efficiency.

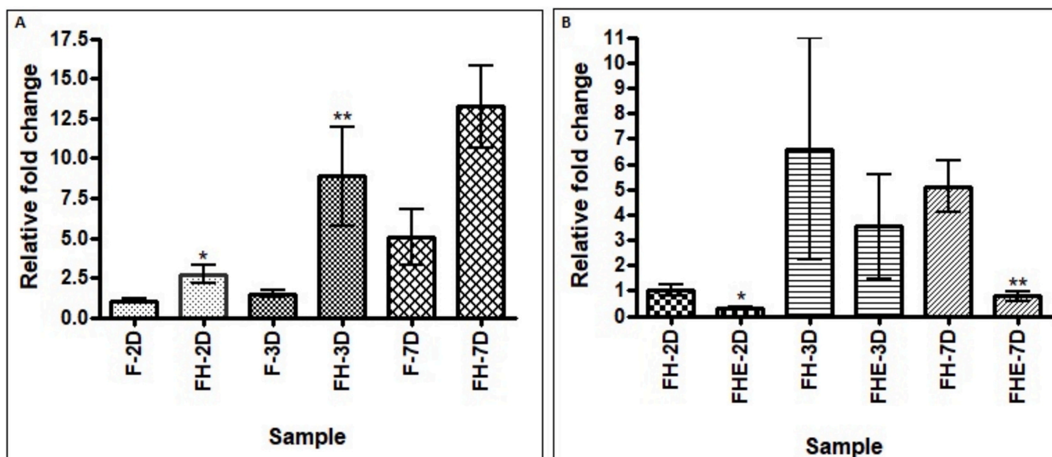

Fig. 11. A) Modulation of gene expression in *Microsporum canis* supplemented with hair. The reference used was the 2-day conidial solution. B) Modulation of gene expression in *M. canis* treated with *S. lancea* ethanolic leaf extract (0.312 mg/mL). The reference used was the supplemented conidial solution without the extract at 2-days. Significant difference $p < 0.05$ (*); $p < 0.01$ (**) in expression within each incubation period. F = untreated fungi; FH = fungi + hair; FHE = fungi + hair + *S. lancea* extract (0.312 mg/mL); 2D = 2 days; 3D = 3 days and 7D = days.

The current study revealed the ethanolic leaf extract of *S. lancea* to consist of flavonoid (sakuranetin) and phenolic (gentisic acid) compounds amongst other compounds. Flavonoids inhibit fungal growth through various underlying mechanisms of action including disruption of the plasma membrane, synthesis of RNA and disruption of the efflux-mediated pumping system [80,81]. There is limited information on the effects of flavonoids and plant extracts on the sulfite pump. In a study by Silva et al. [82], an alkaloid (riparin III (RIP3)) was found to decrease sulfite formation during the growth of *Trichophyton rubrum* in an L-cysteine-rich environment, suggesting that RIP3 affects the sulfite pump. Another flavonoid (Riparin II) was found to interfere with the Ssu1 protein [83]. This suggests that *S. lancea* ethanolic leaf extract could be affecting the sulfite pump. In comparison to the two studies mentioned above, the target could be the Ssu1 protein rather than *SSU1* gene expression. A high concentration of cysteine is required for the upregulation of the *SSU1* gene for the progression of the infection [51,75]. This is achieved by a successful initial cleavage of S—S bridges by the Ssu1 protein. If this protein is inhibited, concentrations of cysteine remain unchanged, therefore regulation of the *SSU1* gene also remains the same.

5. Conclusions

The findings of this study provide validation of the traditional usage of the selected plants in treating dermatophytosis as all the ethanolic extracts showed antifungal activity against the three dermatophytes. This study provides the first report of the anti-dermatophytic activity and cytotoxicity of the ethanolic extract of *S. lancea* on normal human keratinocytes and the selected fungal species. The ethanolic extract of *S. lancea* could be used in combination with *M. comosus* to reduce the cytotoxic effects of *S. lancea*. The antifungal activity of *S. lancea* could be attributed to the presence of sakuranetin, especially, against *T. mentagrophytes*. The findings of this study suggest the plasma membrane and sulfite efflux pump as some of the targets of the *S. lancea* ethanolic leaf extract. This study revealed promising outcomes in the discovery of an alternative treatment for dermatophyte infections *in vitro* and encourages further exploration of the studied leaf extract. Due to the moderate cytotoxicity of *S. lancea*, investigation of irritancy should be done to determine whether the plant extract will show negative effects when used in a clinical setting as part of a formulation. In addition, *S. lancea* could be evaluated in combination with conventional antifungals to determine whether the concentration can be reduced and whether the activity could be potentiated by the addition of the extract.

Ethical approval

All experimental work was approved by the Research Ethics Committee of the Faculty of Natural and Agricultural Sciences, University of Pretoria (Ethics number: NAS224/2020).

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CRediT authorship contribution statement

Murunwa Madzinga: Conceptualization, Investigation, Methodology, Data curation, Formal analysis, Project administration, Writing – original draft. **Mammoloro Boitshoko L. Malefo:** Methodology, Writing – review & editing. **Chris van der Merwe:** Methodology, Writing – review & editing. **Marco Nuno De Canha:** Methodology, Formal analysis, Writing – review & editing. **Ashish Wadhwani:** Methodology. **Namrita Lall:** Funding acquisition, Resources, Supervision, Writing – review & editing. **Quenton Kritzinger:** Supervision, Project administration, Resources, Writing – review & editing.

Declaration of competing interest

The authors declare that there is no conflict of interest.

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Data availability

Data will be available upon reasonable request.

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