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Optimizing extraction of *Pelargonium sidoides* roots: Impact of ethanol concentration on biological activity of extracts

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ABSTRACT

Pelargonium sidoides DC. (Geraniaceae) is an important indigenous medicinal plant in South Africa, historically employed by various ethnic groups to treat respiratory and gastrointestinal ailments. The proprietary herbal tincture, Umckaloabo®, utilizes an ethanolic extract (EPs® 7630) from the roots of *P. sidoides*, and has demonstrated effectiveness in alleviating symptoms of respiratory infections.

P. sidoides roots contain numerous highly oxygenated coumarins and phenolic metabolites, notably the marker compound umckalin. While Umckaloabo® is prepared using 11 % ethanol extraction, the South African commercial market predominantly employs 60 % ethanol extraction due to its consistent umckalin yield and antimicrobial activity. The effects of these extraction methods on chemical composition and *in vitro* pharmacological activity remain poorly understood.

This study confirmed superior antibacterial and antifungal activity in the 60 % ethanol extracts prepared from six root samples compared to the 11 % ethanol extracts. MIC values for both Gram-negative and Gram-positive bacteria ranged from 0.078 to 2.5 mg/mL in the 60 % extracts. Remarkably, two of the root extracts exhibited excellent to very good activity against *C. albicans* and *C. neoformans* with MIC values of 0.039 and 0.078 mg/mL, respectively. A positive correlation was identified between total minimum inhibitory concentration (MIC) levels and polyphenol content, rather than umckalin levels. The influence of these extracts on the pro-inflammatory cytokine IL-6 was assessed. Both 60 % and 11 % root extracts of *P. sidoides* at 100 μg/mL significantly reduced IL-6 production, with the 60 % extracts demonstrating a more pronounced effect ($p = 0.008$).

Statistical analysis revealed significant differences between 11 % and 60 % ethanol extractions in terms of average MIC overall, average Gram-negative MIC, average Gram-positive MIC, fungal MIC, and IL-6 levels. Extracts with higher polyphenol values exhibited superior antimicrobial activity and antioxidant potential, suggesting that polyphenol content may serve as a more reliable indicator of antimicrobial activity than umckalin levels. Additionally, polyphenol levels in the roots may vary with altitude and other environmental factors, warranting further research.

1. Introduction

The commercially available *Pelargonium sidoides* (Pelargonium) root extract, EPs® 7630, also known by the trade name Umckaloabo®, has been found to be effective for symptomatic relief of the common cold (Lizogub et al., 2007). The license for the medicinal use of the extracts from the roots of *P. sidoides* was given in December 2005 by the German Federal Institute for Drugs and Medicinal Devices (Brendler and van Wyk. 2008; Conrad et al., 2007). Moreover, it has been authorised for use in several countries in Europe, Asia, Australia, and Central and South America (Panara et al., 2022). In South Africa, there are also various Pelargonium products on the market, of which Linctagon®, from Nativa (Pty) Ltd, South Africa, is the best known.

The remarkable complexity of metabolites present in *Pelargonium sidoides* and *P. reniforme* is evident through the presence of an extensive array of compounds, including coumarins, coumarin glycosides, coumarin sulfates, flavonoids, proanthocyanidins, phenolic acids, and phenylpropanoid derivatives, as elucidated by Kolodziej (2007). Amongst these compounds, the antibacterial and antiviral effects are primarily attributed to gallic acids and various other phenolic compounds, while immunomodulatory activity is believed to stem from a

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combination of phenolic compounds and the numerous coumarins, such as umckalin and its derivatives.

Schotz and Noldner (2007), along with Schotz et al. (2008), provide a comprehensive analysis of the constituents within EPs® 7630, an aqueous ethanolic extract derived from the roots of *Pelargonium sidoides*. Notably, the extraction process yields a distinct profile of constituents, diverging significantly from those obtained via non-polar solvents. EPs® 7630 can be categorized into six main groups of constituents: purine derivatives (2 %), benzopyranones (2 %), peptides (10 %), carbohydrates (comprising both monomeric and oligomeric forms, totalling 12 %), minerals (12 %), and substituted and unsubstituted oligomeric prodelphinidins (40 %).

Some of the substances in *P. sidoides* have not yet been identified, and tests carried out with the individual chemical constituents versus the whole extract have shown that the whole extract possesses an optimal therapeutic effect. The isolated individual compounds displayed very weak effects if any (Kolodziej and Scholtz, 2003). The ingredient responsible for the overall biological effects of *P. sidoides* is, therefore, currently unknown, and it is speculated that the activity may result from the synergistic action of the various constituents. One theory is that the coumarins have better activity in the presence of the polyphenols, which increases their bioavailability (European Medicines Agency EMA, 2018; Kolodziej, 2007).

The mode of action for EPs® 7630 is multi-faceted and includes antiviral and cyto-protective properties, antibacterial activity, as well as secretomotory properties (Brendler and van Wyk, 2008). Summaries of all the *in vitro* studies undertaken up to 2014 have been published (Moyo and van Staden, 2014). There have also been approximately 30 clinical studies conducted in the last 25 years on EPs® 7630 (Brendler et al., 2020). The antimicrobial action of *P. sidoides* extracts is partially attributed to the presence of coumarins and other phenolic compounds (Kayser and Kolodziej, 1997; Colling et al., 2010).

The active drug substance in EPs® 7630 is a patented dry root extract with a herb-extract ratio of 1:8–10. The extract solvent used is 11 % ethanol m/m, but the reason for the selection of that percentage is not known. The German pharmaceutical company, Schwabe, held a patent on the extraction until 2010 when it was revoked, opening up the market to other companies also making extracts of *P. sidoides* roots. The reason for this was that the patent allowed a monopoly of the market, circumventing the European Patent conventions which disallow patents on life (patents on plant varieties). Since the European Medicines Agency (EMA) approved EPs® 7630, most companies currently follow the 11 % extraction method due to the large body of research conducted on this extract.

For the last decade in South Africa, 60 % ethanol was used for extraction due to the optimal extraction of bioactive constituents from the pelargonium root and to reduce the risk of microorganism contamination. The 60 % ethanol extraction is faster and the yield of umckalin is better than 11 % ethanol extraction (personal communication, Donnie Malherbe, Afriplex Pty Ltd, February 2023, Afriplex®PS70 Active Pharmaceutical Ingredient - Afriplex®PS70 Active Ingredient | Pelargonium Herbal Extract (afriplexps70.com)). Also, *P. sidoides* roots are rich in gallic acid and proanthocyanidins, and antioxidant herbs are generally extracted by using the HAB4a method, which stipulates 10–20 % of root material and 60–62 % ethanol. The HAB (Homeopathisches Arzneibuch) is the specialised procedure laid down in the German homeopathic Pharmacopoeia, or HAB, monograph for antioxidant-rich plants (personal communication, Wendy Clapham, Parceval, March 2023).

In South Africa, *P. sidoides* roots are wild harvested in the Eastern Cape and Free State provinces, and extracts are prepared from the mature red roots of this plant. According to the European Pharmacopoeia, the root has to contain a minimum of 2 % of tannins, expressed as pyrogallol ($C_6H_6O_3$), and no other guidance exists on the quality control of the resulting extract. Only the drug extraction ratio is stipulated, which can lead to huge variations in final product strength. It is common practice in South Africa to test the umckalin levels in the root and then to retest after extraction to standardise the umckalin level. This has limitations as, due to natural variations in the roots found from different areas, the equivalent dried root per dosage cannot be consistently and accurately calculated. In the literature, there are instances where the umckalin level is suggested to be controlled for quality purposes and, in some instances, so are gallic acids (as part of the polyphenols) (Viljoen et al., 2022; Othman, 2009). Recent research has also reinforced the importance of the polyphenol quantity of plants in general, as these compounds play a major role in the antioxidant and antibacterial effect of the extracts (Alara et al., 2021). There are still doubts regarding the most suitable solvent for polyphenol extraction, but it is generally believed that solvents of higher polarity often perform best in terms of polyphenol extraction because of the high solubility of polyphenols in such solvents (Alara et al., 2021).

In the public domain, there is a paucity of information regarding the rationale behind utilizing an 11 % m/m ethanol extraction method for *Pelargonium sidoides*. Historically, this approach has been employed by Schwabe, and all their clinical studies have been conducted using an aqueous ethanol solution. Notably, in 2014, the South African Health Products Regulatory Authority (SAHPRA) introduced new regulations for complementary medicines, mandating the registration of all products within a specified timeframe. It is important to highlight that, presently, there are no specific requirements in place for Western herbal medicines like *Pelargonium sidoides* in South Africa. Consequently, the industry's recourse has been to refer to overseas regulatory approvals, such as those by the European Medicines Agency (EMA), which endorses the use of 11 % ethanol based on the extensive work conducted on the market leader. Therefore, it becomes imperative to elucidate the potential disparities between the previously employed 60 % ethanol extraction and the 11 % stipulated by overseas regulatory authorities. To shed light on this matter, a comparative assessment of 11 % and 60 % extractions versus biological activity was undertaken. This analysis serves as a valuable contribution to the scientific discourse surrounding *Pelargonium sidoides* extraction methods.

To measure the biological activity in relation to the relevant constituents, the umckalin levels of *P. sidoides* root was determined using Ultra-Performance Liquid Chromatography–High-Resolution Mass Spectrometry (UPLC—HRMS). Quantification of markers takes place with the relevant standards. Additionally, the total polyphenol percentage can be quantified via the Folin-Ciocalteu method. The assessment of high molecular-weight proanthocyanidins via LCMS can be challenging due to their size and complexity (Fraser et al., 2012). While they have been widely studied for their potential health benefits, it is often challenging to accurately measure and quantify individual components and is therefore more practical to rather measure total polyphenol content, as this provides a more comprehensive description of the polyphenolic content of a sample.

There are currently no guidelines from the South African Health Products Regulatory Authority (SAHPRA) for the use of *P. sidoides* for the South African market. To date, no published data exist describing the differences between using 11 % or 60 % ethanol for extraction on the chemical composition and biological activity of *P. sidoides* roots. In light of the regulatory requirements, it is imperative to establish the difference between 11 % and 60 % ethanol extracts, as well as gain some insight into the total polyphenol content and the effect this has on biological activity.

2. Materials and methods

2.1. Plant sample selection

Different *P. sidoides* roots, selected on commercial availability, were collected in December 2019 by Afrigetics Botanicals. Six

Table 1
P. sidoides root collection overview.

Sample name	PS1 (*n* = 1)	PS2 (*n* = 1)	PS3 (*n* = 1)	PS4 (*n* = 1)	PS5 (*n* = 1)	PS6 (*n* = 1)
Region	Alice	Alice	Alice	Wepener	Wepener	Wepener
Altitude	382 m	710 m	434 m	1673 m	1496 m	1583 m
Annual rainfall (2019)	500 mm	500 mm	500 mm	784.9 mm	784.9 mm	784.9 mm
Location	Verge east of a river-bed south of Calderwood	South West facing hillock north of Jani Primary school	Along the upper section of a South West facing hill	Bottom of South West facing verge, in the pockets of lower growing grass	Plains beneath a west facing hill	South West facing slope
Biome	Grassland	Grassland	Grassland/Albany thicket	Grassland	Grassland	Grassland
Vegetation type	Flood plain	Hill slope	Hill slope	Hill slope	Plain	Hill slope
Soil type	Gravel with clay content	Loam with dark clay	Fine sand with clay present	Loam with high clay present	Sandy with high clay present	Dark loam with high clay present
Lithology	Sandstone	Sandstone	Sandstone	Sandstone	Sandstone	Sandstone
Exposure aspect	South West Gentle slope, fully exposed	South West gentle slope, fully exposed	South West gentle slope, fully exposed	South West gentle slope, fully exposed	Slight incline, fully exposed	Slight incline, fully exposed
Biotic effect	Abandoned land, grazed	Abandoned land, grazed	Thicket grazed by goat and cow	Thicket grazed by goat and cow	Grassland grazed by goat and cow	Grassland grazed by goat and cow
Plant features	Some flowers were in flower and in bud	No flowers	No flowers	One flower in whole population	Floriferous population with some seeds	Previous season's dried leaves present
Flowers	Yes	No	No	No	Yes	No
Fruit	No	No	No	No	Yes	No
Plant height	10 cm	10 cm	10 cm	10 cm	2 cm	10 cm
GPS Coordinates						
Latitude	32°51′17.4″S	32°44′57.7″S	32°43′39.9″S	29°42′18.2″S	29°41.57′57.5″S	29°44′5″S
Longitude	26°43′9.9″E	26°56′56.5″E	26°51′14.1″E	27°2′16.6″E	27°0′41.2″E	27°4′7.4″E

representative root samples from different areas were collected, with three batches being sourced from Alice (Eastern Cape) and three batches from Wepener (Free State). The conditions around the sample conditions were recorded. In all the experiments, the roots from the different areas were labelled PS1 to PS6 (Table 1). After harvesting, the roots were washed, chopped and dried. Root samples were ground to a powder using a grinder and kept in sealed glass jars in a dark at room temperature.

2.2. Plant extraction and preparation

2.2.1. Pelargonium root samples PS1-PS6

Ten-gram portions of each ground root sample were extracted with 100 mL of 11 % or 60 % ethanol (AR grade, Minema, South Africa) in glass jars. The mixture was vigorously shaken and sonicated in a sonicating bath for at least 30 min. The jars were placed overnight on a shaker and shaken at 100 rpm to allow for maximum extraction. The mixture was filtered through Whatman No. 1 filter paper and the resulting liquid extract was collected into pre-weighed glass vials. The procedure was repeated twice on the same plant material. The combined extracts were then concentrated by evaporation under a stream of cold air. The dried extracts were then weighed, and the yield was calculated by dividing the extracted mass by the initial dry plant mass. Dried extracts were stored at 4 °C until further use.

2.2.2. Microbial strains

Microbial strains implicated in causing respiratory tract infections were selected for the study and were obtained from the American Type Culture Collection (ATCC). These bacterial strains included: *Escherichia coli* ATCC 25922 (Gram-negative), *Pseudomonas aeruginosa* ATCC 27853 (Gram-negative), *Klebsiella pneumoniae* ATCC 700603 (Gram-negative), *Klebsiella aerogenes* ATCC 13048 (Gram-negative), *Staphylococcus aureus* ATCC 29213 (Gram-positive) and *Bacillus cereus* ATCC 21366 (Gram-positive). Fungi were *Candida albicans* (ATCC 10231) and *Cryptococcus neoformans* (ATCC 32045).

2.2.3. In vitro *antimicrobial serial microdilution assay*

The antibacterial and antifungal assays were carried out using microdilution methods as described by Eloff (1998) and Masoko et al. (2007). All the bacteria in this study were prepared by inoculating a single colony of each bacterial strain from a Mueller Hinton agar plate into sterilised Mueller Hinton (MH) broth and grown overnight in a shaking incubator. All fungal strains were inoculated into Sabouraud Dextrose (SD) broth from SD agar and placed in a shaking incubator for 24 h. Each culture was adjusted to a McFarland standard No 1 (equivalent to 3×10^8 cfu/mL). One hundred microliters of sterile water were added to each well of sterile 96-well microplates. Plant samples (100 μl) re-suspended to 10 mg/mL in not more than 20 % acetone were added to the first well of the microplates and then serially diluted along the ordinate. Gentamicin (Virbac) and amphotericin B (Sigma) were used as positive controls for the bacteria and fungi, respectively, while acetone and water were used as negative controls. Subsequently, 100 μl of each of the appropriately adjusted bacterial or fungal cultures were added to the wells of the microplates. Microbes were exposed to extract final concentrations ranging from 2.50 to 0.02 mg/mL. The microplates were incubated at 37 °C for bacteria and 30 °C for fungi for 24 h. To each well of the overnight incubated microplates, 40 μl of 0.2 mg/mL p-iodonitrotetrazolium (INT, Sigma) was added to the bacteria plates. As regards the fungal plates, 40 μl of INT were added prior to incubation. The plates were further incubated at 37 °C for 30 min before reading the MIC for the bacteria, while readings were taken after 24 and 48 h for the fungi. The last well with clear inhibition of bacterial and fungal growth was recorded as the MIC.

2.2.4. Anti-biofilm assay

2.2.4.1. Inhibition of bacterial biofilm formation. The inhibition of biofilm formation by extracts of the plants was assessed via the protocol of Sandasi et al. (2010) and Mohsenipour and Hassanshahian (2015). A strong biofilm former, *K. aerogenes* ATCC 13048 was used for this

study. Two biofilm development stages were investigated, namely the prevention of biofilm attachment (T_O) and destruction of 24 h-old pre-formed biofilm (T_{24}). The biofilm was allowed to preform for either 0 h (T_O) or 24 h (T_{24}) before the addition of plant extracts at a final concentration of 1 mg/mL. For the T_O study, 100 μl of the respective standardised bacterial culture (OD_{590} = 0.02 equivalent to 1.0×10^6 CFU/mL) prepared in Tryptone Soy Broth (TSB) was inoculated into sterile flat-bottomed 96-well microtitre plates followed by addition 100 μl of the plant samples and incubation for 24 h at 37 °C without shaking. For T_{24}, 100 μl of the standardised cultures were pre-incubated for 24 h for biofilm growth, before the addition of plant extracts. Gentamicin (Virbac) served as the positive control, while acetone and sterile water served as negative controls. After 24 h incubation, the modified crystal violet staining (CVS) assay (Sandasi et al., 2010) was performed to quantify the biofilm biomass.

2.2.5. Crystal violet staining assay

Following the incubation described in Section 2.2.4.1, the wells were carefully emptied, and plates were washed at least three times with sterile distilled water to remove unattached or loosely attached cells. The plates were air-dried and then oven-fixed at 60 °C for 45 min. Adhered cells were stained with 100 μl of 0.1 % crystal violet solution for 20 min at room temperature. The excess stain was rinsed off by washing the plates at least five times with water. Thereafter, the biofilm biomass was evaluated semi-quantitatively by re-solubilising the crystal violet stain bound to the adherent cells with 150 μl of 100 % ethanol to destain the wells. The absorbance of the plates was read at 590 nm using a microplate reader (Epoch ™ Microplate Spectrophotometer) after careful and gentle shaking. The mean absorbance (OD_{590nm}) of the sample was determined, and results were expressed as percentage inhibition using the equation below (Sandasi et al., 2010).

$$\%\ inhibition\ = 100\ \times\ \frac{OD_{Negative\ Control} - OD_{Sample}}{OD_{Negative\ Control}}$$

2.2.6. In vitro *cytotoxicity assay*

The cytotoxicity test was carried out by screening the acetone extracts of the six root samples against African green monkey kidney (VERO) cells (ATCC® CCL-81™) using the tetrazolium-based colorimetric (MTT) assay described by Mosmann (1983) and modified by McGaw et al. (2007). The cell lines were maintained in Minimal Essential Medium (MEM, Separation Scientific SA (Pty) Ltd, South Africa) supplemented with 0.1 % gentamicin (Virbac) and 5 % foetal calf serum (FCS, Capricorn Scientific GmbH, South America) at 37 °C in a 5 % CO_2 incubator till confluence. Cells of a sub-confluent culture were harvested and centrifuged at 700 x g for 7 min and re-suspended in MEM to 10^5 cells/mL. Cell suspension (100 μl) was pipetted into each well of columns 2 to 11 of a tissue culture grade sterile 96 well microtitre plate and only MEM (200 μl) was pipetted in columns 1 and 12 to minimise the "edge effect" and maintain humidity. The plates were incubated for 24 h at 37 °C in a 5 % CO_2 incubator.

Different concentrations of the extracts prepared in the complete medium were added to the plates in quadruplicate with 2 repeats (n = 8). The microtitre plates were then incubated at 37 °C in a 5 % CO_2 incubator for 48 h. Doxorubicin (Pfizer Laboratories) and acetone served as the positive and negative controls, respectively. The contents of the cells were discarded and washed with phosphate-buffered saline and replaced with 200 μl of fresh MEM. Then, 30 μl MTT (Sigma, stock solution of 5 mg/mL in phosphate-buffered saline (PBS)) was added to each well, and the plates were incubated for a further 4 h at 37 °C. The medium was aspirated, and MTT formazan crystals were dissolved with 50 μl dimethyl sulphoxide (DMSO). The plates were shaken gently on an orbital shaker to allow the formazan to dissolve. The amount of MTT reduction was measured immediately by detecting absorbance in a microplate reader at a wavelength of 570 nm. The half-maximal lethal concentration (LC_{50}) value was calculated. Selectivity index (SI) values for antimicrobial activity were calculated using the formula SI = LC_{50}/MIC.

2.2.7. Total antibacterial activity (TAA)

The TAA of extracts was calculated by dividing the quantity extracted from 1 g of plant material with the MIC values obtained against bacteria or fungi in mg/mL (Eloff, 2001). TAA indicates the amount in mL to which the amount extracted from 1 g of plant material can be diluted and still inhibit the growth of the test organism.

2.2.8. Immunomodulatory assay

2.2.8.1. Cell culture and treatment with samples. RAW 264.7 macrophage cells ATCC® TIB-71™ (Rockville, MD, USA) were grown at 37 °C with 5 % CO_2 in Dulbecco's Modified Eagle Medium (DMEM) containing 4.5 g/L glucose and 4 mM L-glutamine (Hyclone™), supplemented with 10 % FCS (Capricorn Scientific GmbH, South America) and 1 % penicillin/streptomycin (PS). One hundred μL of a cell suspension (1×10^6 cells/mL) of RAW 264.7 cells were seeded in a 96-well microtitre plate and incubated overnight at 37 °C with 5 % CO_2 to allow for attachment. After this, lipopolysaccharide (LPS; 100 μl of 1 μg/mL solution) prepared in the complete medium was added to the wells to stimulate cytokine production. After an hour, 100 μl (100 μg/mL) of the samples were added to the LPS stimulated wells. Quercetin served as positive control. After 24 h incubation in a CO_2 incubator, the supernatant was collected and stored at −70 °C.

2.2.8.2. Measurement of cytokine (IL-6). Commercial kits (Biocom Africa) were used according to the manufacturer's guidelines to determine the level of cytokines (Interleukin-6, or IL-6) in the supernatant obtained above.

2.2.9. Anti-inflammatory assay: nitric oxide production inhibition assay in raw 264.7 macrophages

2.2.9.1. Culturing of cells and sample testing. Macrophages were cultured in 75 cm^2 flasks in DMEM containing L-glutamine supplemented with 10 % FCS and 1 % penicillin/streptomycin/fungizone (PSF; Sigma-Aldrich, South Africa) at 37 °C with 5 % CO_2. Cells were seeded at a concentration of 10^6 cells/mL in 96-well microtitre plates and incubated overnight at 37 °C with 5 % CO_2 to allow for attachment. Then the cells were activated by the addition of 1 μg/mL of LPS (Sigma-Aldrich, South Africa) followed by the addition of different concentrations (100, 50, 25, and 12.5 μg/mL) of the extracts. Indomethacin served as the positive control. Cells were incubated for 24 h at 37 °C and 5 % CO_2.

2.2.9.2. Nitrite measurement. Following incubation for 24 h, an equal volume of cell culture supernatants and Griess reagent (Sigma-Aldrich, South Africa) were added to wells of a 96-well plate, incubated for 15 min, and the absorbance was measured at 550 nm using a microtitre plate reader (Epoch Biotek). The percentage nitric oxide (NO) inhibition was calculated based on the ability of extracts to inhibit NO formation by macrophages compared with the control (cells treated with LPS only). The half-maximal inhibitory concentration (IC_{50}) was calculated from the equation obtained by plotting the concentrations with corresponding% NO inhibitory values.

2.2.9.3. Determination of cell viability. To verify that the NO inhibitory activity observed from the extract was not due to general toxicity to the macrophages, a cytotoxicity assay using 3-(4,5-dimethythiazol-2-yl)−2,5-diphenyl tetrazolium bromide (MTT; Inqaba biotec, South Africa) was done (Mosmann, 1983). After the removal of supernatant from the RAW 264.7 macrophages, the cells were washed with PBS,

and fresh culture medium MTT solution (5 mg/mL) was added to all wells, and the plates were incubated for 4 h. The medium was discarded, and DMSO (50 μl) was added to each well to dissolve the formed formazan salts. The absorbance was read using a microplate reader (Biotek Synergy, USA) at 570 nm. The percentage cell viability for each sample was calculated by comparing the absorbance in the plant extract-treated wells to the untreated cells.

2.2.10. Antioxidant activity: 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging assay

The simple, quick and widely used method to measure the ABTS radical scavenging activity of the acetone crude extracts of the plants (Re et al., 1999) was used. Ascorbic acid served as a positive control, methanol as a negative control, while extract without ABTS was the blank. ABTS (160 μl) was mixed with extracts (40 μl) at different concentrations, and incubated for 5 min in the dark, followed by measuring the absorbance at 734 nm using a microplate reader (Epoch, BioTek). The percentage of radical scavenging activity was calculated using the formula below:

$$\%\ Scavenging\ Activy = 100 - 100.\frac{Absorbance\ of\ Sample - Absorbance\ of\ Sample_{Blank}}{Absorbance\ of\ Control - Absorbance\ of\ Control_{Blank}}$$

The IC_{50} values of samples were obtained from the graph plotted as inhibition percentages against the concentrations. Each test was done in triplicate.

2.3. Quantitative chemical analysis

2.3.1. Ultra-Performance liquid chromatography–high-resolution mass spectrometry (UPLC—HRMS) chromatographic conditions

Separation was performed on a Kinetix®1.7 μm EVO C18 100 Å (2.1 × 100 mm) column using a step gradient elution scheme which consisted of H_2O (0.1 % formic acid) and MeOH (0.1 % formic acid) as solvent A and solvent B, respectively. The gradient elution method was optimised as follows: an isocratic hold (0–0.1 min) followed by a linear increase to 100 % solvent B (0.1–17 min) prior to a 1 min column wash step (17–18 min) at 100 % solvent B before re-conditioning at the initial conditions prior to the start of the next run. The column temperature was kept constant at 50 °C, and the flow rate was set at 0.5 mL/min, giving a total run time of 20 min. Injection volumes were set at 5 μl. To ensure unbiased analysis, the samples were analysed in both ESI positive and ESI negative ionisation modes, and conducted in separate analyses.

2.3.2. UPLC-HRMS instrumentation and MS conditions

Separation and detection of umckalin was performed using a Waters® Synapt G2 high-definition mass spectrometry (HDMS) system (Waters Inc., Milford, Massachusetts, United States of America (USA)). The system was operated with MassLynx™ (version 4.1) software (Waters Inc., Milford, Massachusetts, USA) for data acquisition and processing.

An internal lock mass control standard, 2 ng/μL solution leucine enkephalin (*m/z* 555.2693), was directly infused into the source through a secondary orthogonal electrospray ionisation (ESI) probe allowing intermittent sampling.

The internal control was used to compensate for instrumental drift throughout the duration of the runs. The instrument was calibrated using sodium formate clusters and the Intellistart functionality (mass range 112.936 – 1 132.688 Da). A resolution of 20 000 at *m/z* 200 (full width at half maximum (FWHM)) and mass error within 0.4 mDa were obtained. The source conditions were as follows: the capillary voltage for ESI was 2.6 kV and 2.0 kV for positive and negative mode ionisation, respectively. The source temperature was set at 120 °C, the sampling cone voltage at 25 V, the extraction cone voltage at 4.0 V and cone gas (nitrogen) flow at 10.0 litre per hour (L/Hr). The desolvation temperature was set at 350 °C with a gas (nitrogen) flow of 600.0 L/Hr.

2.3.3. Data acquisition for the UPLC-HRMS analysis

Quantitative data-independent acquisition (DIA) was done using two simultaneous acquisition functions with low and high collision energy (mean squared error (MSE) approach) with a quadrupole-time-of-flight (QTOF) detector. Fragmentation was performed using high-energy collision-induced dissociation (CID). The fragmentation energy was set at 2 V and 3 eV for the trap and collision energy, respectively. The ramping was set from 3 to 4 eV and 20 to 40 eV for the trap and transfer collision energy, respectively.

Mass spectral scans were collected every 0.3 s, and the raw data were collected in the form of a continuous profile. Mass-to-charge ratios (*m/z*) between 50 and 1 200 Da were recorded.

2.3.4. Quantification of umckalin in Pelargonium sidoides roots

Quantification of umckalin was carried out with the use of a 5-point calibration curve prepared for umckalin (R = 0.9996). The umckalin standard working solution was prepared by accurately weighing 1.0 mg of umckalin (Toronto Research Chemicals) and dissolving it in 1.0 mL of MeOH (ROMIL-SpS™). Standard solutions were prepared by serial dilution of the original working solutions and used for subsequent quantification of the extracts. The dried down extracts were reconstituted in MeOH and centrifuged and diluted prior to analysis. The concentration of umckalin for each sample was determined on a dry weight basis (*%w/w*) of the MeOH extract.

2.3.5. Quantification of total polyphenol content in P. sidoides

The Folin-Ciocalteu assay was conducted based on the assay of Biglari et al. (2008) with some modifications. Briefly, a Folin-Ciocalteu working solution was prepared from the stock solution by diluting the 10 x stock with ddH_2O. A 20 ul aliquot of reconstituted methanol extract was placed in a microtitre plate together with 100 ul of Folin-Ciocalteu working solution, and the mixture was shaken on a microtitre plate shaker. Thereafter, 80 μL of a 7.5 % (*%w/v*) sodium carbonate solution prepared in ddH2O was added, mixed and incubated at room temperature for 60 min. The absorbance was measured at 760 nm with a Multiscan Ascent plate reader from AEC Amersham (Kelvin, South Africa). Water was used as a blank, and a standard curve was constructed with a 1 mg/mL GA solution prepared in ddH_2O, linear concentration range from 7.8 to 125 μg/mL. Results were expressed as mg gallic acid equivalents (GAE)/g of sample.

2.4. Statistical analysis

Statistical analysis was performed using Microsoft Excel (version 16.0) and JMP Pro-software (version 16.0) for Windows, developed by SAS Institute Inc (North Carolina, US). Normal distribution was assessed using an Anderson-Darling test. A mixed model for repeated measures analysis was conducted to investigate the difference between the extraction method (11 % versus 60 %) ethanol with the individual samples as random effects. Descriptive statistics were reported as the median (interquartile range), and the significance level, alpha (α), was set at 0.05.

3. Results and discussion

3.1. Plant extract yields

The percentage yields of the dried root material extracted by the two different solvents (11 % and 60 % ethanol) are represented in Table 2.

The 11 % ethanol extracts had the highest yield for root samples PS2 to PS5, whereas roots PS1 and PS2 had the highest yield

Table 2
Pelargonium root extract sample yields.

Root sample	Yield (%)	
	60 % ethanol	11 % ethanol
PS1 (n = 1)	2.55	0.53
PS2 (n = 1)	4.20	3.33
PS3 (n = 1)	1.05	4.33
PS4 (n = 1)	1.20	4.15
PS5 (n = 1)	1.73	3.65
PS6 (n = 1)	0.53	3.20
Average	**1.88**	**3.20**

following 60 % ethanol extraction. This was not statistically significant (p = 0.2). There were quite large differences between the two solvent extracts per sample observed. The yield and composition of secondary metabolites within a species may vary between plants from different geographical locations and may be influenced by environmental and genetic differences (White et al., 2008).

Based on the observed trends in terms of% yield, the 11 % ethanol extraction provided a greater yield, although it needs to be noted that the chemical composition is not known. It is well reported that the plant roots contain a high amount of water-soluble compounds such as polysaccharides, peptides and proteins, and this might explain why a more polar extract has a greater yield (Schoetz et al., 2008; Kolodziej, 2007). This result supports the extraction technique used commercially by Schwabe, i.e. using 11 % ethanol to obtain a superior quantitative yield of more water-soluble compounds.

3.2. Quantification of umckalin and polyphenols

The umckalin values of the root samples were characterised using UPLC—HRMS and total polyphenols were determined by the Folin-Ciocalteu method. The data are presented in Table 3.

The root sample PS2 had the highest polyphenol levels for both the 11 % and 60 % extracts, however the other root samples collected from the Eastern Cape (PS1-PS3) were lower in total polyphenols. The root samples collected from the Free State generally had higher levels of total polyphenols. The Free State roots were growing in an area of higher altitude, and the influence of this on umckalin and polyphenol content is yet to be determined. It is clear that the geographical area from which the root is harvested affects levels of the extracted constituents. Roots collected from plants growing at higher altitudes have higher polyphenol levels in this study. *P. sidoides* extracts mediate their effects mainly via two classes of compounds: oxygenated coumarins and prodelphinidins that belong to the polyphenols group (Janecki et al., 2011). It has been stated before that umckalin values are higher in areas of lower rainfall (White, 2006). It would therefore make sense that roots collected from the Eastern Cape have higher umckalin levels. There is no data available on the effect that rainfall and altitude have on the polyphenol levels of *P. sidoides* roots. Although the sample size was small, it can be seen from Table 3 that the polyphenol levels varied quite considerably. It is assumed with a bigger sample size that this difference in polyphenol levels would be more clearly elucidated.

3.3. Antimicrobial activity

3.3.1. Antibacterial activity

The MIC values for the PS1 to PS6 root 11 % extracts ranged between 0.313 – 2.5 mg/mL for both Gram-negative bacteria and Gram-positive bacteria (Table 4). The MIC values for the PS1 to PS6 root 60 % extracts ranged between 0.078 – 2.5 mg/mL for Gram-negative bacteria and 0.313 – 2.5 mg/mL for Gram-positive bacteria.

P. aeruginosa, with a mean MIC of 0.078 mg/mL (Table 4), was the most susceptible of the Gram-negative bacteria against root extract PS2 (11 %). Of the Gram-positive bacteria, *S. aureus* was the most susceptible to the 60 % extracts of both PS2 and PS5.

Table 3
Umckalin and total polyphenol levels in the 11 % versus 60 % extracts.

Extract		Umckalin (mg/kg)	Total Polyphenols (mg/kg)
11 %	PS 1	9 288	23
	PS 2	3 741	210
	PS 3	19 228	17
	PS 4	8 442	118
	PS 5	26 249	86
	PS 6	4 412	175
AVE*		**11 893**	**105**
60 %	PS 1	7 771	27
	PS 2	3 916	321
	PS 3	18 175	58
	PS 4	12 764	204
	PS 5	6 441	229
	PS 6	23 702	127
AVE*		**12 128**	**161**

* = average.

Eloff (2021) classified the antimicrobial activity of plant extracts in the following way: outstanding activity MIC < 0.02 mg/mL, excellent activity MIC = 0.021–0.04 mg/mL, very good activity MIC = 0.04 –0.08 mg/mL, good activity MIC = 0.081 –0.16 mg/mL, average activity MIC = 0.161–0.32 mg/mL and weak activity MIC > 0.32 mg/mL. Based on this classification, all the ethanol extracts of the investigated root samples had average activity against at least two of the bacteria tested. Of all the samples tested, root sample labelled PS2 had the best average activity against all the organisms, indicating that it had the broadest spectrum of antibacterial efficacy. The 60 % root extracts were overall more active than the 11 % extracts. This could be due to higher average levels of umckalin and total polyphenols in the 60 % versus 11 % extracts (Table 3. It was stated by Jekabsone et al. (2019) that the likely distinct antibacterial activity of *P. sidoides* extracts can be ascribed to the polyphenolic ingredients.

The commercial market-leading *P. sidoides* preparation, EPs® 7630, has been reported to moderately inhibit the growth of *Streptococcus pyogenes, Proteus mirabilis, Staphylococcus aureus, E. coli, Streptococcus pneumoniae, Haemophilus influenza, Staphylococcus epidermidis* and some other Gram-positive and Gram-negative organisms as summarised by Moyo and Van Staden (2014). The MIC values found in this study, therefore, correspond with previous reports of *P. sidoides* extracts exhibiting only moderate direct antibacterial activity (EMA, 2018). The demonstrated activity cannot adequately describe the documented clinical efficacy.

In this study, the PS2 and PS5 root extracts had the highest TAA against *P. aeruginosa* (269 and 167 mL/g for 60 % and 833 and 51 mL/g for 11 %), respectively. The highest TAA of 833 mL/g (Table 5) was produced by the 11 % extract against *P. aeruginosa,* which indicates that the extract amountobtained from 1 g of PS2 root can be diluted in 833 mL of solvent and still inhibit the growth of the organism.

The TAA is useful for determining the most suitable plant extract for compound isolation and bioprospecting. Samples PS2 and PS5 would be the best choice to choose as starting materials to make extracts for the development of therapeutic preparations based on their activity for both the 11 % and 60 % ethanol extractions. This shows the importance of selecting the optimum starting roots before extracting.

In all the antimicrobial assays the effect observed was only moderate in comparison with the positive controls. When comparing the antibacterial effect of *P. sidoides* to the reference drug gentamicin, it is important to note that gentamicin is a potent antibiotic prescribed for a wide range of bacterial infections. *P. sidoides* only had moderate

Table 4
Antibacterial activity of the extracts (prepared using 11 % and 60 % ethanol) against Gram-positive and Gram-negative bacteria implicated in causing respiratory tract infections.

Organisms	MIC (mg/mL)												
	60 % Extracts						11 % Extracts						
	PS1	PS2	PS3	PS4	PS5	PS6	PS1	PS2	PS3	PS4	PS5	PS6	Gentamicin
E. coli	1.250	0.313	1.250	0.625	0.625	0.625	2.500	0.313	2.500	0.625	0.625	0.625	0,0008
P. aeruginosa	1.250	0.156	2.500	0.156	0.156	0.156	0.313	**0.078**	2.500	0.156	0.313	1.250	0.0003
K. pneumoniae	0.625	0.625	2.500	0.625	0.625	0.625	1.250	0.313	2.500	0.625	0.625	0.625	0.0313
K. aerogenes	1.250	0.313	2.500	0.625	0.313	0.625	2.500	0.625	>2.5	2.500	1.250	0.625	0.0156
S. aureus	1.250	0.156	2.500	0.625	0.313	0.625	1.250	0.313	2.500	1.250	0.313	0.313	0.0004
B. cereus	2.500	0.313	2.500	0.625	0.313	0.625	2.500	2.500	2.500	>2.5	1.250	2.500	0.0039
Average	**1.354**	**0.312**	**2.291**	**0.546**	**0.39**	**0.546**	**1.718**	**0.690**	**2.5**	**1.276**	**0.729**	**0.989**	

Bold = very good activity (MIC = 0.04 – 0.08 mg/mL), good activity (MIC = 0.081 – 0.16 mg/mL); average activity (MIC = 0.161–0.32 mg/mL; weak activity (MIC > 0.32)

Table 5
Percentage yields and TAA of the extracts against bacteria.

Organism	TAA* (mL/g)											
	60 % Extracts						11 % Extracts					
	PS1	PS2	PS3	PS4	PS5	PS6	PS1	PS2	PS3	PS4	PS5	PS6
E. coli	20	134	8	19	42	13	20	208	17	58	51	73
P. aeruginosa	20	269	4	77	167	51	160	833	17	234	102	36
K. pneumoniae	41	67	4	19	42	13	40	208	17	58	51	73
K. aerogenes	20	134	4	19	83	13	20	104	NA	15	26	73
S. aureus	20	269	4	19	83	13	40	208	17	29	102	145
B. cereus	10	134	4	19	83	13	20	26	17	NA	26	18
% yield	2.55	4.2	1.05	1.2	1.73	0.53	0.53	3.33	4.33	4.15	3.65	3.2
Average	**21.8**	**167.8**	**4.7**	**28.7**	**83.3**	**19.3**	**50**	**264.5**	**17**	**78.8**	**59.7**	**69.7**

*TAA: total antibacterial activity, or volume (mL) to which the extract obtained from one g of plant material can be diluted and still be able to inhibit bacterial growth.
NA not applicable.

antibacterial effect and therefore cannot be seen as a substitute for conventional antibiotics. *P. sidoides* also showed some moderate antifungal activity and again had a much weaker effect than amphotericin B. Amphotericin B is a very potent and effective antifungal drug. The reference is usually a pure compound so this effect is expected.

3.3.2. Antifungal activity

The extracts of PS2 and PS5 had excellent to very good activity against *C. albicans* and *C. neoformans* with MIC of 0.039 and 0.078 mg/mL, respectively (Table 6). For *C. neoformans* the TAA was 1077 and 167 mL/g for 60 % and 833 and 51 mL/g for 11 % (Table 7).

In this study the 60 % ethanol extracts had better antifungal activity than the 11 % extracts. This is in line with previous studies which showed the antifungal activity of the polyphenol fraction in *P.* sidoides roots (Latté and Kolodziej, 2000).

A few *in vitro* studies have assessed the antifungal activity of *P. sidoides* extracts, mostly against *C. albicans*, but have not investigated any mechanisms behind inhibition (Conrad et al., 2007; Moyo et al., 2013). One study found that *Pelargonium* extracts had significant antifungal activity against various *Candida* species, including *C. albicans*. The MIC values ranged from 6.25 to 12.5 mg/mL (Shokoohinia et al., 2017). Recent work by Samie et al. (2019) highlighted the necessity to perform further work on the antifungal activities of *Pelargonium*. The broad-spectrum activity may present another treatment option in scenarios where alternatives are crucial.

3.3.3. Anti-biofilm activity

3.3.3.1. Prevention of cell attachment: anti-biofilm activity / anti-adhesion. All extracts had no activity except for the 11 % and 60 % ethanol extracts of PS2, which showed poor activity (see Supplementary 3, Table 1). The results of antibiofilm (ABF) potential against *Klebsiella aerogenes* are presented in Table 8 below. Extracts or fractions resulting in inhibition above 50 % were considered to have good ABT activity (++) while those with inhibition between 0 and 50 % indicated poor ABF activity (+) and the values <0 (-) were regarded as no inhibition or enhancement of biofilm development and growth. This indicates that *P. sidoides* extracts do not inhibit biofilms of *K. pneumoniae*.

The better anti-adhesive activity of the 60 % ethanol plant extracts was also substantiated by Maisuria et al. (2016) who showed that cranberry-derived proanthocyanins, a subset of polyphenols, can interfere with the N-acyl homoserine lactone-mediated quorum sensing of *Pseudomonas aeruginosa*. Moreover, proanthocyanins have also been shown to compromise adhesion to host cells by mimicking cell surface signalling (Michaelis et al., 2011). Some authors have proposed the hypothesis that proanthocyanins might increase bacterial membrane permeability and cause indirect metabolism to decrease due to adenosine triphosphate (ATP) and other intracellular metabolic loss (Di Pasqua et al., 2007; Thapa et al., 2012).

Adhesion of pathogenic bacteria to the host cell surface is a crucial event in colonisation and infection (Kolodziej, 2011). Inhibition of the microbial docking process to epithelial cells at an early stage would be effective in protecting host cells from infection. Using fluorescent-labelled group A-streptococci and viable human laryngeal cells (HEp-2) as a model, flow cytometric measurements showed prominent (by up to 45 % compared with untreated cells) anti-adhesive and anti-invasive capabilities of EPs® 7630 in a concentration-dependent manner (<30 ug/mL). Notably, inhibition of streptococcal adhesion to host cells was only evident when the bacteria were pre-treated with EPs® 7630, indicating that the interaction of the anti-adhesive principle occurred only with the bacterial outer membrane surface and not with the binding sides at the epithelial surface. This correlates with our data, as well as with the extracts containing the highest amounts of polyphenols showing some activity.

Table 6
Antifungal activity of the extracts.

Organism	MIC (mg/mL)												
	60 % Extracts						11 % Extracts						
	PS1	PS2	PS3	PS4	PS5	PS6	PS1	PS2	PS3	PS4	PS5	PS6	Amphotericin B
C. albicans	1.250	0.313	1.250	0.625	0.625	0.625	2.500	0.625	2.500	2.500	1.250	1.250	0.0078
C. neoformans	0.625	**0.039**	1.250	0.156*	0.156*	0.625*	1.250	**0.078**	2.500	2.500	0.625	0.625	0.0313
Average	**0.938**	**0.176**	**1.250**	**0.391**	**0.391**	**0.625**	**0.625**	**0.352**	**2.500**	**2.500**	**0.938**	**0.938**	**0.020**

Bold = significantly active (0.04 – 0.08 mg/mL), good activity (0.081 – 0.16 mg/mL).

Table 7
Percentage yields and TAA of the extracts.

Organism	Total activity (mL/g)											
	60 % Extracts						11 % Extracts					
	PS1	PS2	PS3	PS4	PS5	PS6	PS1	PS2	PS3	PS4	PS5	PS6
C. *albicans*	20	134	8	19	42	13	20	104	17	15	26	36
C. *neoformans*	41	1077	8	77	167	13	40	833	17	15	51	73
% yield	2.55	4.2	1.05	1.2	1.73	0.53	0.53	3.33	4.33	4.15	3.65	3.2
Average TAA	**30.5**	**605.5**	**8**	**48**	**104.5**	**13**	**30**	**468.5**	**17**	**15**	**38.5**	**54.5**

Table 8
Cytotoxicity (LC_{50} in mg/mL) and selectivity index (SI) of the plant extracts against Vero kidney cells.

	Extract	LC_{50}	*E. coli*	*P. aeruginosa*	*K. pneumoniae*	*K. aerogenes*	*S. aureus*	*B. cereus*	*C. albicans*	*C. neoformans*
60%	PS 1	>1	>0.8	>0.8	>**1.6**	>0.8	>0.8	>0.4	>0.8	>**1.6**
	PS 2	0.2	0.5	**1.1**	0.3	0.5	**1.1**	0.5	0.5	**25.6**
	PS 3	>1	>0.8	>0.4	>0.4	>0.4	>0.4	>0.4	>0.8	>0.8
	PS 4	0.3	0.5	**2.2**	0.5	0.5	0.5	0.5	0.5	**2.2**
	PS 5	0.3	0.5	**2.1**	0.5	1.0	**1.0**	**1.0**	0.5	**2.1**
	PS 6	0.2	0.3	**1.3**	0.3	0.3	0.3	0.3	0.3	0.3
11 %	PS 1	>1	>0.4	>**3.2**	>0.8	>0.4	>0.8	>0.4	>0.4	>0.8
	PS 2	0.4	**1.2**	**4.7**	**1.3**	0.6	**1.2**	0.2	0.6	**4.7**
	PS 3	>1	>0.4	>0.4	>0.4	>0.4	>0.4	>0.4	>0.4	>0.4
	PS 4	0.6	1.0	**4.1**	1.0	0.3	0.5	<0.3	0.3	0.3
	PS 5	>1	>**1.6**	>**3.2**	>1.6	>0.8	>**3.2**	>0.8	>0.8	>**1.6**
	PS 6	0.2	0.2	0.1	0.2	0.2	0.5	0.06	0.1	0.2

SI values >1 regarded to be more toxic to bacterial cells than mammalian cells.

The mechanism proposed to explain the observed activity in this study is the prevention of bacterial attachment and biofilm formation by prodelphinidin-rich proanthocyanins, as is known to occur in *P. sidoides* (Jekabsone et al., 2019).

3.4. Cytotoxicity and selectivity indices

In this study, the 60 % extracts of PS1 and 3, as well as the 11 % extracts of PS1, 3, and 5 had the lowest cytotoxicity against Vero kidney cells as their LC_{50} values were more than 1 mg/mL, the highest tested concentration (Table 9). In terms of the selectivity index (SI) values, compared to the other samples tested, the 60 % and 11 % extracts of PS2 and PS5 had SI values of at least 1 against three or more of the tested pathogens (Table 8). The selectivity index (SI) was noted in the 11 % extract of root PS2 as 4.7 against *P. aeruginosa* and *C. neoformans*, while the 60 % extract of the same root sample had an outstanding SI value of 25.6 against *C. neoformans*. This indicated that root PS2 is a promising option to explore further for product or extract development (Vonthron-Sénécheau et al., 2003).

According to Vonthron-Sénécheau et al. (2003), an SI value greater than or equal to 10 implies that biological efficacy is not due to *in vitro* cytotoxicity but there is a possible therapeutic use. SI values above 4 obtained in this study suggest that the extracts may have potential as a treatment for *C. neoformans* infections. A higher selectivity index indicates that the extract is effective in inhibiting the growth of the microorganism at a lower concentration than that at which it is toxic to human cells, implying that it has potential therapeutic value, but this needs to be investigated using *in vivo* experiments.

While there is limited research available on the specific antifungal activity of *P. sidoides* against *C. neoformans*, the available studies (Samie et al., 2019) suggest that Pelargonium extracts may have some potential as antifungal agents against a range of fungi, including other pathogenic species. Further research is needed to fully understand the spectrum and mechanisms of the antifungal activity of Pelargonium extracts and their potential as alternative or complementary treatments for fungal infections.

Our study showed that the bioactivity of *P. sidoides* roots against *C. neoformans* varies depending on the region where the plant was grown, and higher activity correlates with higher polyphenol levels. The importance of polyphenols has been shown before in literature for ingredients that are rich in proanthocyanidins such as grapeseed extract (GSE). Recently, high antifungal activity of GSE, rich in polymeric flavan-3-ols, against a broad panel of human fungal pathogens was demonstrated against *Candida* spp., *C. neoformans* and dermatophytes (Simonetti et al., 2014).

3.5. Anti-inflammatory activity

The effects of the plant extracts on the inhibition of nitric oxide production were determined in LPS-stimulated RAW 264.7 macrophages. The extracts and the positive control, indomethacin,

Table 9
Inhibitory activities of the different extracts on NO production and cell viability in LPS-activated RAW 264.7 macrophages.

Extract	60 %				11 %			
	Conc. (μg/mL)	NO inhibition (%)	Macrophage cell viability (%)	IC_{50} (μg/mL)	Conc. (μg/mL)	NO inhibition (%)	Macrophage cell viability (%)	IC_{50} (μg/mL)
PS1	100	85.42	91.36	60.67[b]	100	11.76	105.78	>100*
	50	44.49	90.54		50	4.36	100.54	
	25	14.25	99.09		25	1.46	104.86	
	12.5	3.77	105.01		12.5	−0.46	112.79	
PS2	100	59.07	99.57	84.47[c]	100	35.63	87.25	>100*
	50	30.52	97.34		50	23.33	81.23	
	25	13.87	99.02		25	12.21	94.15	
	12.5	9.90	101.56		12.5	16.13	110.85	
PS3	100	42.20	97.30	>100*	100	25.98	97.25	>100*
	50	13.22	88.98		50	21.37	89.60	
	25	5.67	90.88		25	20.43	91.99	
	12.5	4.90	98.22		12.5	18.99	104.29	
PS4	100	40.77	96.11	>100*	100	27.53	90.91	>100*
	50	6.93	93.47		50	14.22	87.46	
	25	3.29	100.57		25	12.74	98.57	
	12.5	0.48	107.62		12.5	10.96	102.81	
PS5	100	60.49	104.38	82.72[c]	100	55.48	96.13	87.62[c]
	50	29.20	100.52		50	33.70	91.58	
	25	23.27	101.78		25	26.64	95.26	
	12.5	22.18	102.24		12.5	21.98	102.09	
PS6	100	71.40	88.69	64.85[b]	100	46.23	70.31	>100*
	50	39.38	83.24		50	25.96	72.00	
	25	25.39	89.00		25	19.67	81.22	
	12.5	22.40	99.64		12.5	18.16	86.52	
Indomethacin	100	91.61	81.16	23.27[a]	100	91.61	81.16	23.27[a]
	50	70.66	98.86		50	70.66	98.86	
	25	45.47	117.42		25	45.47	117.42	
	12.5	37.46	126.29		12.5	37.46	126.29	

Note: Superscript letters a-c represent statistical significance between IC_{50} values, and values with different letters are significantly different at $p<0.05$. * Also values >100 μg/mL (highest tested concentration) were not part of the analysis.

inhibited NO production in a concentration-dependent manner at the tested concentrations of 100, 50, 25 and 12.5 μg/mL (Table 9). The IC_{50} of indomethacin was 23.27 μg/mL, which is much lower than that found with the extracts. The 60 % extracts of PS1 and PS6 had the lowest IC_{50} values, implying that a lower concentration is needed for 50 % inhibition of NO activity. This parameter was not statistically significant (p = 0.0827) amongst the two extractant groups.

3.6. Immunomodulatory activity interleukin-6 (IL-6)

The influence of the different root extracts on the levels of the cytokine IL-6, a pro-inflammatory cytokine, was determined. Both 60 % and 11 % root extracts of *P. sidoides* at 100 μg/mL significantly reduced IL-6 production in LPS-induced RAW 264.7 mouse macrophages compared to LPS-induced cells only; however, the best effect was seen with the 60 % extracts versus 11 % extracts (p = 0.008). The best performing root samples were PS2 and PS5 (Table 10).

Table 10
Statistical analysis of inhibition of IL-6 production for the *Pelargonium sidoides* root extracts.

60 %	Mean IL-6 concentration (pg/mL)*	11 %	Mean IL-6 concentration (pg/mL)
PS1	97.04 ± 3.62	**PS1**	1 536.97 ± 5.07
PS2	42.21 ± 1.45	**PS2**	908.22 ± 84.06
PS3	933.32 ± 97.83	**PS3**	1199.79 ± 36.96
PS4	297.91 ± 28.26	**PS4**	1579.50 ± 18.84
PS5	80.13 ± 1.45	**PS5**	408.08 ± 17.39
PS6	77.05 ± 2.90	**PS6**	897.45 ± 47.10
LPS + cells	2 688.40 ± 247.84	**LPS + cells**	2 688.40 ± 247.84
Quercetin	545.43 ± 38.85	**Quercetin**	545.43 ± 38.85

Values >100 μg/mL (highest tested concentration) were not part of the analysis.
*Il-6 concentrations were measured in picograms (pg) per millilitre.

The results of this study correlate with those reported by Jekabsone et al. (2019) who reported that extracts with the highest polyphenol values had the strongest effect on IL-6.

The extract EPs® 7630 has been shown to stimulate the release of NO, type I interferon (IFN), and different cytokines involved in host defence mechanisms (Kolodziej, 2011; Witte et al., 2015, 2020). In athletes, EPs® 7630 modulated the production of secretory immunoglobulin A in saliva, both interleukin-15 and interleukin-6 in serum, and interleukin-15 in nasal mucosa (Luna et al., 2011). EPs® 7630 was found to prevent asthma attacks provoked by rhinovirus in children, probably by interfering with IL-6, IL-8 and IL-16 mediated inflammation (Tahan and Yaman, 2013). The data available suggest that EPs® 7630 induces NO release in a broad spectrum of infectious conditions and, importantly, in therapeutically relevant doses. EPs® 7630 has also been reported to be rich in proanthocyanidins, which supports the notion of high polyphenol levels being associated with anti-inflammatory effects (Keck et al., 2021).

3.7. Antioxidant activity

3.7.1. The 2, 2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging assay

In the ABTS assay, IC_{50} values ranged from 0.28 to 259.06 μg/mL compared to Trolox, the positive control, which had IC_{50} of 9.92 mg/mL (Table 11). PS2 had the best antioxidant activity in this assay. This root extract also had the highest polyphenol levels, which are likely to be linked to the antioxidative effect and possibly the antimicrobial effect. Strong antioxidant activity has been shown for gallic acid and proanthocyanidins by other authors (Bagchi et al., 2000; Cos et al., 2002; Spranger et al., 2008; Sroka and Cisowski, 2003).

Table 11
Antioxidant activity (ABTS) of *Pelargonium sidoides* extract samples (11 % and 60 %).

11 %	**ABTS IC_{50} (μg/mL)**	**60** %	**ABTS IC_{50} (μg/mL)**
PS1	122.23	PS1	96.70
PS2	10.82	PS2	0.28
PS3	259.06	PS3	81.21
PS4	70.14	PS4	11.05
PS5	99.12	PS5	13.74
PS6	24.85	PS6	19.85
AVE	**97.70**	**AVE**	**37.14**
Trolox	9.92	Trolox	9.92

3.8. Statistical comparison of the main markers and biological activity of the 11 % and 60 % extracts

A Repeated Measures Regression Analysis (Mixed Model) was conducted using JMP to evaluate the effects of the different extraction methods (11 % versus 60 %) for all the different values obtained for biological activity and the selected chemical markers. The effects were not uniform across all the variables. When assessing the fit least squares model, it could be seen that the following components differed statistically significantly between the 11 % and 60 % ethanol extractions, namely average MIC overall, average Gram-negative MIC, average Gram-positive MIC, fungal MIC and IL-6 levels (see Supplementary 4, Table Summary fit least squares model ordered by goodness of fit).

The proanthocyanins fraction of polyphenols (PACN) possesses a range of biological activities, including anti-inflammatory and antibacterial (Aron and Kennedy, 2008). The capacity to suppress inflammation is related to both strong antioxidant and metalloproteinase-inhibiting properties (Raudone et al., 2019; Balalaie et al., 2018), whereas antibacterial efficiency is achieved due to the prevention of bacterial adhesion and biofilm formation, as well as direct antibacterial activity (Shahzad et al., 2015).

When assessing the biological activity compared to the concentrations of the main constituents, the extracts with the highest polyphenol values had the best antimicrobial activity and antioxidant potential. Extraction with a higher concentration of ethanol extracted more bioactive constituents, but a much larger sample size is required to further establish the significance of this finding.

The root PS1 extract with higher umckalin levels had a better activity with respect to immune modulatory activity, but more data is required to substantiate this supposition. The root PS2 generally had better activity regarding MIC, IL-6 inhibition and antioxidative properties.

In all the biological activity assays, the 60 % extracts had better activity than the 11 % extracts. It has been shown previously that *P. sidoides* extracts contain multiple bioactive compounds, and using 60 % ethanol appears to enhance extraction of the main constituents assessed in this study and biological activity too. Our study further substantiated the importance of the presence of high levels of polyphenols and not just umckalin in the mode of action of *P. sidoides*. This is supported by recent key publications (Montenegro-Landivar et al, 2021; Annunziata et al., 2020; Keck et al., 2021), showing that the complex polyphenol structure is responsible for the observed effect. The efficacy of a natural product may be due to two or more constituents acting synergistically, and their respective bioactivities often diminish or disappear upon separation into individual chemical entities (Moyo et al., 2013). In particular, polyphenolic compounds in plant extracts are known to increase the bioavailability of anti-infective constituents in phytopharmaceuticals thereby enhancing their anti-infective properties (Wagner, 2011). Natural products can also work synergistically with antibiotics, thereby improving their overall pharmacokinetic properties against resistant bacterial strains (Schmidt et al., 2007). Table 12 provides a summary of the biological activity results as well as the umckalin and total polyphenol levels for all extracts, namely PS1 to PS6, 11 % and 60 % extracts. In this table the average MIC values for Gram-positive (Gram +) and Gram-negative (Gram -) bacteria, respectively, were used as a measure of antibacterial efficacy to allow general comparisons to be made.

For all the parameters that differed statistically significantly, the 60 % ethanol extracts performed superiorly in terms of overall MIC, antimicrobial efficacy against both Gram-positive and Gram-negative bacteria as well as fungi, and also regarding inhibition of IL-6 production. The other components differed, but these were not statistically significant.

3.9. Correlations

There was no correlation between the umckalin levels and total polyphenol levels for the different samples (see Supplementary 2, Fig. 1). Seemingly, these concentrations are sample dependent with a high variability. The sample itself seems to plays a more important role in the levels of the phytochemical constituents than the actual extraction method.

The overall MIC was negatively correlated with the total polyphenol values (the higher the total polyphenols, the lower the MIC) with R^2=0.66 and (p = 0,0012) (see Supplementary 2, Fig. 2).

The latest research substantiates that the polyphenols are a better gauge of antibacterial activity than just the widely accepted marker compound, umckalin, in *P. sidoides* (Jekabsone et al., 2019; Keck et al., 2021). This can be ascribed to a few reasons, including the structural

Table 12
Summary of all biological activity and umckalin and total polyphenol levels for all extracts.

		Average MIC overall (mg/mL)	**Gram-negative (mg/mL)**	**Gram-positive (mg/mL)**	**Fungi (mg/mL)**	**ABTS IC_{50} (μg/mL)**	**IL-6 (pg/mL)**	**NO Assay IC_{50} (μg/mL)**	**Umckalin (mg/kg)**	**Total polyphenols (mg GAE/kg)**
11 %	PS 1	1.76	1.64	1.88	1.88	122.23	1537	>100 μg	9288	23.07
	PS 2	0.61	0.33	1.41	0.35	10.82	908	>100 μg	3741	210.29
	PS 3	2.50	2.50	2.50	2.50	259.06	1200	>100 μg	19,228	16.79
	PS 4	1.45	0.98	1.25	2.50	70.14	1580	>100 μg	8442	118.30
	PS 5	0.78	0.70	0.78	0.94	99.12	408	86.62 μg	26,249	86.34
	PS 6	0.98	0.78	1.41	0.94	24.85	897	>100 μg	4412	174.75
	AVG	**1.35**	**1.16**	**1.54**	**1.52**	**97.70**	**1088**		**11,893**	**104.92**
60 %	PS 1	1.25	1.09	1.88	0.94	96.70	97	60.67 μg	7771	27.05
	PS 2	0.28	0.35	0.24	0.18	0.28	42	84.47 μg	3916	321.07
	PS 3	2.03	2.19	2.50	1.25	81.21	933	>100 μg	18,175	57.96
	PS 4	0.51	0.51	0.63	0.39	11.05	298	>100 μg	12,764	203.94
	PS 5	0.39	0.43	0.31	0.39	13.74	80	82.72 μg	6441	228.76
	PS 6	0.57	0.51	0.63	0.63	19.85	77	64.85 μg	23,702	126.95
	AVG	**0.838**	**0.847**	**1.029**	**0.629**	**37.14**	**254.6**		**12,128**	**106.96**

diversity of polyphenols and their multiple phenolic hydroxyl groups, making them highly reactive towards bacterial cells. They can interact with bacterial lipids, proteins and carbohydrates, interfering with the bacterial membrane, cell wall and cellular processes, ultimately leading to bacterial death. In contrast, coumarins have a less reactive structure without any phenolic hydroxyl groups, making them less capable of interacting with bacterial cells. Secondly, studies have shown that the antibacterial activity of polyphenols is related to their antioxidant activity. Polyphenols possess antioxidant properties that can neutralise reactive oxygen species (ROS) generated by bacteria during infection. By doing so, they prevent bacterial growth and the formation of biofilms, inhibiting bacterial colonisation and virulence. Coumarins do not possess significant antioxidant activity, making them less effective as antibacterial agents (Resende et al., 2013; Cao and Sofic, 2006; Lin et al., 2005). The market leader EPs® 7630 mainly consists of polyphenolic compounds, and the oligo and polymeric proanthocyanidins based on gallocatechin and epigallocatechin moieties account for 40 % of the extract (Schötz and Nolder, 2007; Schoetz et al., 2008). These prodelphinidins are present in enormous structural variety, making it difficult to attribute activity to a single compound or even to a group of compounds, hence the reasoning to rather determine the total polyphenol values. Proanthocyanins have received some attention as antimicrobial compounds (Theisen and Muller, 2012). Because of the volume of this component in the extract (40 %) and the biological activity ascribed to it, it is recommended that the total polyphenol levels are assessed when selecting *P. sidoides* roots for the production of commercial preparations and when evaluating extracts.

4. Conclusion

P. sidoides is an important medicinal plant from southern Africa with worldwide commercial value. Over the last decade in South Africa, the only parameter assessed in the extracts for quality control was the content of umckalin, and all commercial extracts were standardised based on the umckalin level. Most commercial extraction processes in this country thus focus on extracting the optimal amount of umckalin. The data from this study strongly suggest that the polyphenol concentration in dried *P. sidoides* root material is a more rational indicator of possible biological and pharmaceutical activity than umckalin concentrations. Confounding effects include the fact that the roots are wild harvested with no indication of the age of the roots, amongst other parameters. *P. sidoides* is wild harvested from the Eastern Cape and Free State provinces of South Africa, and even though there have been studies evaluating the effect of rainfall on umckalin values, there has been no evaluation of the impact of altitude on constituent levels in the root. More work on the influence of geographical location is still required, but preliminary information from this research indicates that higher altitudes might be correlated with higher polyphenol levels. Altitude may enhance the polyphenol content, but more research is needed. In our study 60 % ethanol extracted higher levels of constituents with better biological activity compared to 11 % ethanol. The root sample PS2 had the best activity overall, highlighting the variation in biological activity between root samples collected from different areas. A combination of using the most bioactive starting material available with the optimal extraction process will ensure best results in terms of a commercially viable extract. However, it does seem that the root source plays the biggest role in determining the quality of the resulting extract.

Further investigations are warranted to comprehensively assess the roots of *Pelargonium sidoides* sourced from diverse geographical locations and altitudes, with a specific focus on constituent levels. Presently, post-wild harvesting, the assessment primarily revolves around umckalin levels, and subsequent dried root sales are often based on this metric alone. It stands to reason that incorporating the evaluation of total polyphenol levels into this process could prove invaluable. This multifaceted approach aims to discern patterns and provide an additional metric for assessing the quality of roots. By doing so, we enhance our capacity to gauge and monitor the diverse attributes of these roots. This endeavour holds great potential in advancing our understanding and quality control measures within the context of *Pelargonium sidoides* roots.

These conclusions are of a preliminary nature and require more statistically relevant data derived from larger sample sizes, but they are a departure from current industrial and scientific wisdom and bear focused consideration. This is especially important as there are currently no data available from a South African regulatory perspective on how *P. sidoides* should be handled by pharmacists in the industry. The advantages of investigations in this area include more commercially economical and biologically active extracts, as well as a better chance of eventually selecting optimum quality roots that are able to reduce symptoms and duration of illness due to colds and flu. It is also likely that the extracts contain a range of metabolites that contribute to a healthy outcome through synergistic action.

Conflict of interest

JVW is employed by Nativa (Pty) Ltd, South Africa and the company provided funds to support the research but had no influence on the design of experiments or analysis of results.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: The project was funded largely by Nativa (Pty) Ltd and the first author is employed by the company. Nativa had no part in influencing the design of the study and had no influence in analysis of the results.

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Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.sajb.2023.09.058.

References

Alara, O.R., Abdurahman, N.H., Ukaegbu, C.I., 2021. Extraction of phenolic compounds: a review. Curr. Res. Food Sci. 4, 200–214. https://doi.org/10.1016/j.crfs.2021.03.011.

Aron, P.M., Kennedy, J.A., 2008. Flavan-3-ols: nature, occurrence and biological activity. Mol. Nutr. Food Res. 52 (1), 79–104. https://doi.org/10.1002/mnfr.200700137.

Annunziata, F., Pinna, C., Dallavalle, S., Tamborini, L., Pinto, A., 2020. An overview of coumarin as a versatile and readily accessible scaffold with broad-ranging biological activities. Int. J. Mol. Sci. 21 (13), 4618. https://doi.org/10.3390/ijms21134618.

Bagchi, D., Bagchi, M., Stohs, S.J., Das, D.K., Ray, S.D., Kuszynski, C.A., Joshi, S.S., Pruess, H.G., 2000. Free radicals and grape seed proanthocyanidin extract: importance in human health and disease prevention. Toxicology 148 (2–3), 187–197. https://doi.org/10.1016/s0300-483x(00)00210-9.

Balalaie, A., Rezvani, M.B., Basir, M.M., 2018. Dual function of proanthocyanidins as both MMP inhibitor and crosslinker in dentin biomodification: a literature review. Dent. Mater. J. 37 (2), 173–182. https://doi.org/10.4012/dmj.2017-062.

Biglari, F., AlKarkhi, A.F.M., Mat Easa, A., 2008. Antioxidant activity and phenolic content of various date palm *(Phoenix dactylifera)* fruits from Iran. Food Chem. 107 (4), 1636–1641. https://doi.org/10.1016/j.foodchem.2007.10.033.

Brendler, T., van Wyk, B., 2008. A historical, scientific and commercial perspective on the medicinal use of *Pelargonium sidoides* (Geraniaceae). J. Ethnopharmacol. 119, 420–433. https://doi.org/10.1016/j.jep.2008.07.037.

Brendler, T., Al-Harrasi, A., Bauer, R., Gafner, S., Hardy, M.L., Heinrich, M., et al., 2020. Botanical drugs and supplements affecting the immune response in the time of COVID-19: implications for research and clinical practice. Phytotherapy Res. 35 (6), 3013–3031. https://doi.org/10.1002/ptr.7008.

Cao, G., Sofic, E., 2006. Prior studies of antioxidants *in vitro* and *in vivo* show that polyphenols protect against a wide range of diseases. Am. J. Clin. Nutr. 81 (1), 230S–232S.

Colling, J., Groenewald, J.H., Makunga, N.P., 2010. Genetic alterations for increased coumarin production lead to metabolic changes in the medicinally important *Pelargonium sidoides* DC (Geraniaceae). Metab. Eng. 12 (6), 561–572. https://doi.org/10.1016/j.ymben.2010.08.001.

Conrad, A., Jung, I., Tioua, D., Lallemand, C., Carrapatoso, F., Engels, I., Daschner, F.D., Frank, U., 2007. Extract of *Pelargonium sidoides* (EPs® 7630) inhibits the interactions of group A-streptococci and host epithelia *in vitro*. Phytomedicine 14 (6), 52–59. https://doi.org/10.1016/j.phymed.2006.11.018.

Cos, P., Rajan, P., Vedernikova, I., Calomme, M., Pieters, L., Vlietinck, A.J., 2002. *In vitro* antioxidant profile of phenolic acid derivatives. Free Radic. Res. 36 (6), 711–716. https://doi.org/10.1080/10715760290029182.

Di Pasqua, R., Betts, G., Hoskins, N., Edwards, M., Ercolini, D., Mauriello, G., 2007. Membrane toxicity of antimicrobial compounds from essential oils. J. Agric. Food Chem. 55 (12), 4863–4870. https://doi.org/10.1021/jf0636465.

Eloff, J.N., 1998. A sensitive and quick microplate method to determine the minimal inhibitory concentration of plant extracts for bacteria. Planta Med. 64, 711–713. https://doi.org/10.1055/s-2006-957563.

Eloff, J.N., 2001. Antibacterial activity of marula (*Sclerocarya birrea* (A. Rich.) hochst. subsp. *caffra* (Sond.) Kokwaro) (Anacardiaceae) bark and leaves. J. Ethnopharmacol. 76, 305–308. https://doi.org/10.1016/s0378-8741(01)00260-4.

Eloff, J.N., 2021. A proposal towards a rational classification of the antimicrobial activity of acetone tree leaf extracts in a search for new antimicrobials. Planta Med. 87 (10/11), 836–840.

European Medicines Agency (EMA). 2018. Assessment report on *Pelargonium sidoides* DC and/or *Pelargonium reniforme* Curt., radix. Committee on Herbal Medicinal Products (HMPC). EMA/HMPC/444251/2015. https://www.ema.europa.eu/en/documents/herbal-report/final-assessment-report-pelargonium-sidoides-dc/pelargonium-reniforme-curt-radix-revision-1_en.pdf (accessed 30 September 2023).

Fraser, K., Harrison, S.J., Lane, G.A., Otter, D.E., Hemar, Y., Quek, S.Y., Rasmussen, S., 2012. HPLC_MS/MS profiling of proanthocyanidins in teas: a comparative study. J. Food Compos. Anal. 26 (1–2), 43–51. https://doi.org/10.1016/j.jfca.2012.01.004.

Janecki, A., Conrad, A., Engels, I., Frank, U., Kolodziej, H., 2011. Evaluation of an aqueous-ethanolic extract from *Pelargonium sidoides* (EPs® 7630) for its activity against group A-streptococci adhesion to human HE p-2 epithelial cells. J. Ethnopharmacol. 133 (1), 147–152. https://doi.org/10.1016/j.jep.2010.09.018.

Jekabsone, A., Sile, I., Cochis, A., Makrecka-Kuka, M., Laucaityte, G., Makarova, E., Rimondini, L., Bernotiene, R., Raudone, L., Vedlugaite, E., et al., 2019. Investigation of antibacterial and antiinflammatory activities of proanthocyanidins from *Pelargonium sidoides* DC root extract. Nutrients 11 (11), 2829. https://doi.org/10.3390/nu11112929.

Kayser, O., Kolodziej, H., 1997. Antibacterial activity of extracts and constituents of *Pelargonium sidoides* and *Pelargonium reniforme*. Plant Med. 63, 508–510. https://doi.org/10.1055/s-2006-957752.

Keck, T., Strobl, A., Weinhaeusel, A., Funk, P., Michaelis, M., 2021. *Pelargonium* extract EPs® 7630 in the treatment of human corona virus-associated acute respiratory tract infections-a secondary subgroup-analysis of an open-label, uncontrolled clinical trial. Front. Pharmacol. 12. https://doi.org/10.3389/fphar.2021.666546.

Kolodziej, H., 2007. Fascinating metabolic pools of *Pelargonium sodoides* and *Pelargonium reniforme*, traditional and phytomedicinal sources of the herbal medicine Umckaloabo. Phytomedicine 14 (6), 9–17. https://doi.org/10.1016/j.phymed.2006.11.021.

Kolodziej, H., 2011. Antimicrobial, antiviral and immunomodulatory activity studies of *Pelargonium sidoides* (EPs® 7630) in the context of health promotion. Pharmaceuticals 4 (10), 1295–1314. https://doi.org/10.3390/ph4101295.

Kolodziej, H., Scholtz, V., 2003. Umckaloabo phytotherapie. Dtsch. Apoth. Ztg. 143 (12), 1304–1311.

Latté, K.P., Kolodziej, H., 2000. Antifungal effects of hydrolysable tannins and related compounds on dermatophytes, mould fungi and yeasts. Z. Naturforsch. C J. Biosci. 44 (5–6), 467–472. https://doi.org/10.1515/znc-2000-5-625.

Lin, Y.T., Kwon, Y.I., Labbe, R.G., Shetty, K., 2005. Inhibition of food-borne bacteria by plant phenolics. Food Biotechnol. 19 (1), 43–64.

Lizogub, V.G., Riley, D.S., Heger, M., 2007. Efficacy of a *Pelargonium sidoides* preparation in patients with the common cold: a randomized, double blind, placebo-controlled clinical trial. Explore 3 (6), 573–584. https://doi.org/10.1016/j.explore.2007.09.004.

Luna, L.A., Bachi, A.L.L., Novaes e Brito, R.R., Eid, R.G., Suguri, V.M., Oliveira, P.W., et al., 2011. Immune responses induced by *Pelargonium sidoides* extract in serum and nasal mucosa of athletes after exhaustive exercise: modulation of secretory IgA, IL-6 and IL-15. Phytomedicine 18 (4), 303–308. https://doi.org/10.1016/j.phymed.2010.08.003.

Maisuria, V.B., Los Santos, Y.L., Tufenkju, N., Deziel, E., 2016. Cranberry derived proanthocyanins impair virulence and inhibit quorum sensing of *Pseudomonas aeruginosa*. Sci. Rep. 6, 30169. https://doi.org/10.1038/srep30169.

Masoko, P., Picard, J., Eloff, J.N., 2007. The antifungal activity of twenty – four southern African *Combretum* species (Combretaceae). S. Afr. J. Bot. 73 (2), 173–183. https://doi.org/10.1016/j.sajb.2006.09.010.

McGaw, L.J., Steenkamp, V., Eloff, J.N., 2007. Evaluation of *Athrixia* bush tea for cytotoxicity, antioxidant activity, caffeine content and presence of pyrrolizidine alkaloids. J. Ethnopharmacol. 110 (1), 16–22. https://doi.org/10.1016/j.jep.2006.08.029.

Michaelis, M., Doerr, H.W., Citatl, J., 2011. Investigation of the influence of Eps® 7630, a herbal drug preparation from *Pelargonium sidoides*, on replication of a broad panel of respiratory viruses. Phytomedicine 18 (5), 384–386. https://doi.org/10.1016/j.phymed.2010.09.008.

Mohsenipour, Z., Hassanshahian, M., 2015. The effects of *Allium sativum* extracts on biofilm formation and activities of six pathogenic bacteria. J. Microb. 8 (8), E18971. https://doi.org/10.5812/jjm.18971v2.

Montenegro-Landivar, M.F., Tapia-Quirós, P., Vecino, X., Reig, M., Valderrama, C., Granados, M., Cortina, J.L., Saurina, J., 2021. Polyphenols and their potential role to fight viral diseases: an overview. Sci. Total Environ. 801, 149719. https://doi.org/10.1016/j.scitotenv.2021.149719.

Mosman, T., 1983. Rapid colorimetric assay for cellular growth and survival: application to roliferation and cytotoxicity assays. J. Immunol. Method 65 (1–2), 55–63. https://doi.org/10.1016/0022-1759(83)90303-4.

Moyo, M., Aremu, A.O., Gruz, J., Subrtová, M., Szüčová, L., Doležal, K., Van Staden, J., 2013. Conservation strategy for *Pelargonium sidoides* DC: phenolic profile and pharmacological activity of acclimatized plants derived from tissue culture. J. Ethnopharmacol. 149 (2), 557–561. https://doi.org/10.1016/j.jep.2013.07.021.

Moyo, M., Van Staden, J., 2014. Medicinal properties and conservation of *Pelargonium sidoides* DC. J. Ethnopharmacol. 152 (2), 243–255. https://doi.org/10.1016/j.jep.2014.01.009.

Othman, B.A.R.H., 2009. Quality control of *Pelargonium*-containing pharmaceutical preparations. MSc dissertation. Petra University, Amman-Jordan.

Panara, A., Aalizadeh, R., Thomaidis, N.S., 2022. Chemical characterisation of *Pelargonium sidoides* root based on LC-QToF-MS non target screening strategies. Phytochem. Anal. 33 (1), 40–56. https://doi.org/10.1002/pca.3059.

Raudone, L., Vilkickyte, G., Pitkauskaite, L., Raudonis, R., Vainoriene, R., Motiekaityte, V., 2019. Antioxidant activities of *Vaccinium vitis-idaea* L. leaves within cultivars and their phenolic compounds. Molecules 24 (5), 844. https://doi.org/10.3390/molecules24050844.

Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M., Rice-Evans, C., 1999. Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Radical Biol. Med. 26 (9–10), 1231–1237. https://doi.org/10.1016/s0891-5849(98)00315-3.

Resende, A.C., Costa, J., Pereira, C., Tavaria, F.K., 2013. Coumarins as potential antibacterial agents. Phytochem. Rev. 12 (1), 133–149.

Samie, S., Trollope, K.M., Joubert, L., Makunga, N.P., Volschenk, H., 2019. The antifungal and *Cryptococcus neoformans* virulence attenuating activity of *Pelargonium sidoides* extracts. J. Ethnopharmacol. 235, 122–132. https://doi.org/10.1016/j.jep.2019.02.008.

Sandasi, M., Leonard, C.M., Viljoen, A.M., 2010. The *in vitro* antibiofilm activity of selected culinary herbs and medicinal plants against *Listeria monocytogenes*. Lett. Appl. Microbiol. 50, 30–35. https://doi.org/10.1111/j.1472-765X.2009.02747.x.

Schmidt, B.M., Ribnicky, D.M., Lipsky, P.E., Raskin, I., 2007. Revisiting the ancient concept of botanical therapeutics. Nat. Chem. Biol. 3 (7), 360–366. https://doi.org/10.1038/nchembio0707-360.

Schoetz, K., Erdelmeier, C., Germer, S., Hauer, H., 2008. A detailed view on the constituents of EPs® 7630. Planta Med. 74 (6), 667–674. https://doi.org/10.1055/s-2008-1074515.

Schötz, K., Nöldner, M., 2007. Mass spectroscopic characterisation of oligomeric proanthocyanidins derived from an extract of *Pelargonium sidoides* roots (EPs® 7630) and pharmacological screening in CNS models. Phytomedicine 6, 32–39. https://doi.org/10.1016/j.phymed.2006.11.019.

Shahzad, M., Millhouse, E., Culshaw, S., Edwards, C.A., Ramage, G., Combet, E., 2015. Selected dietary (poly)phenols inhibit periodontal pathogen growth and biofilm formation. Food Funct. 6 (3), 719–729. https://doi.org/10.1039/c4fo01087f.

Shokoohinia, Y., Hosseini, R., Sadeghi, E., 2017. Antifungal activity of *Pelargonium sidoides* extract on *Candida albicans*. J. Med. Plants By Prod. 2 (2), 51–55.

Simonetti, G., Santamaria, S.R., D'Auria, F.D., Mulinacci, N., Innocenti, M., Cecchini, F., Pasqua, G., 2014. Evaluation of anti-candida activity of *Vitis vinifera* L. seed extracts obtained from wine and table cultivars. BioMed Res. Int.. https://doi.org/10.1155/2014/127021.

Spranger, I., Sun, B., Mateus, A.M., de Freitas, V., Ricardo-da-Silva, J.M., 2008. Chemical characterization and antioxidant activities of oligomeric and polymeric procyanidin fractions from grape seeds. Food Chem. 108 (2), 519–532. https://doi.org/10.1016/j.foodchem.2007.11.004.

Sroka, Z., Cisowski, W., 2003. Hydrogen peroxide scavenging, antioxidant and anti-radical activity of some phenolic acids. Food Chem. Toxicol. 41 (6), 753–758. https://doi.org/10.1016/s0278-6915(02)00329-0.

Tahan, F., Yaman, 2013. Can the *Pelargonium Sidoides* root extract EPs® 7630 prevent asthma attacks during viral infections of the upper respiratory tract in children? Phytomedicine 20 (2), 148–150. https://doi.org/10.1016/j.phymed.2012.09.022.

Thapa, D., Losa, R., Zweifel, B., Wallace, R.J., 2012. Sensitivity of pathogenic and commensal bacterial from the human colon to essential oils. Microbiology 158, 2870–2877. https://doi.org/10.1099/mic.0.061127-0.

Theisen, L.L., Muller, C.P., 2012. EPs® 7630 (Umckaloabo®), an extract from *Pelargonium sidoides* roots, exerts anti-influenza virus activity *in vitro* and *in vivo*. Antiviral Res. 94 (2), 147–156. https://doi.org/10.1016/j.antiviral.2012.03.006.

Viljoen, A., Chen, W., Mulaudzi, N., Brendler, T., 2022. Chapter 19 - *Pelargonium sidoides*. In: Viljoen, A., Sandasi, M., Fouche, G., Combrinck, S., Vermaak, I. (Eds.), The South

African herbal pharmacopoeia. monographs of medicinal and aromatic plants. Academic Press, Elsevier Inc, pp. 407–446.
Vonthron-Sénécheau, C., Ouattara, M., Trabi, F., Kamenan, A., Anton, R., Weniger, B., 2003. *In vitro* antiplasmodial activity and cytotoxicity of extracts of ethnobotanically selected Ivorian plants. J. Ethnopharmacol. 87, 221–222.
Wagner, H., 2011. Synergy research: approaching a new generation of phytopharmaceuticals. Fitoterapia 82 (1), 34–37. https://doi.org/10.1016/j.fitote.2010.11.016.
White A., 2006. The effect of geography, cultivation and harvest trechnique on the umckalin concentration and growth of *Pelargonium sidoides* (Geraniaceae). MSc dissertation, Rhodes University, South Africa. https://commons.ru.ac.za/vital/access/manager/Repository/vital:4234?site_name=Rhodes%20University
White, A.G., Davies-Coleman, M.T., Ripley, B.S., 2008. Measuring and optimising umckalin concentration in wild-harvested and cultivated *Pelargonium sidoides* (Geraniaceae). S. Afr. J. Bot. 74 (2), 260–267. https://doi.org/10.1016/j.sajb.2007.11.015.
Witte, K., Koch, E., Volk, H.D., Wolk, K., Sabat, R., 2015. The *Pelargonium sidoides* extract EPs® 7630 drives the innate immune defense by activating selected MAP kinase pathways in human monocytes. PLoS One 10 (9), e0138075. https://doi.org/10.1371/journal.pone.0138075.
Witte, K., Koch, E., Volk, H.D., Wolk, K., Sabat, R., 2020. The herbal extract EPs® 7630 increases the antimicrobial airway defense through monocyte-dependent induction of IL-22 in T cells. J. Mol. Med. 98 (10), 1493–1503. https://doi.org/10.1007/s00109-020-01970-3.