Quorum sensing modulation and inhibition in biofilm forming foot ulcer pathogens by selected medicinal plants

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A B S T R A C T

The crisis of antibiotic resistance necessitates the search of phytochemicals as potential antibacterial, anti-quorum sensing and biofilm forming agents. For the present study, fifteen (15) selected medicinal plants were evaluated to inhibit the biological activities of multi-drug resistant (MDR) pathogenic bacteria (Staphylococcus aureus, Escherichia coli, Klebsiella pneumoniae, and Proteus mirabilis) associated with diabetic foot ulcer. Antibacterial activities revealed noteworthy minimum inhibitory concentration (MIC) values ≤1 mg/mL for thirteen (13) out of the sixty (60) plant extracts screened. The potent extracts included Euclea natalensis ethyl acetate (0.25 mg/mL), Aloe ferox methanol (0.5 mg/mL) and Warburgia salutaris aqueous (0.5 mg/mL) extracts. Chemical profiling of the active extracts using gas chromatography-mass spectrometry (GC-MS) identified neophytadiene, guanosine, squalene, cis megastigma-5,8-diene-4-one and sorbitol as prevalent compounds among the active extracts. Anti-quorum sensing activities of E. natalensis (ethyl acetate), A. ferox (methanol) and W. salutaris (aqueous) extracts ranged from 4.81 - 58.34% with E. natalensis (ethyl-acetate) showing the highest activity. Molecular docking against CviR protein showed selected compounds having high docking scores with sorbitol showing the highest score of -7.04 kcal/mol. Warburgia salutaris aqueous extract exhibited the highest biofilm inhibition (73%) against E. coli. Euclea natalensis, Aloe ferox and Warburgia salutaris compounds act as antagonist of N-acyl homoserine lactone (AHL) signaling, thus may serve as candidates in antipathogenic and antibiofilm phytomedicine development for MDR foot ulcer bacterial pathogens.

1. Introduction

Diabetes mellitus (DM) often referred to as diabetes, is a group of metabolic disease whereby patients experience high blood sugar due to insufficient insulin production or the inability of the body to produce insulin [1]. High mortality rates are noted in patients with acute necrotizing soft-tissue infections and chronic osteomyelitis [2]. About 15% of all patients with diabetes have DFU, where 84% have their lower leg amputated and 6% hospitalized due to gangrene and infections mostly colonized by multi-drug resistant (MDR) pathogens [3]. Such pathogens include Escherichia coli, Klebsiella pneumoniae, Morganella morgani, and Proteus mirabilis which dominate and colonize the foot ulcer [4] as well as Streptococcus agalactiae, Streptococcus pyogenes, Streptococcus mitis, Staphylococcus aureus and Enterococcus faecalis. These pathogens express virulence factors like biofilm-forming and others rendering them unmanageable with antibiotics treatment.

Biofilms, which are bacterial communities grow to a critical threshold, coordinate changes in gene expression through an organized cell to cell interaction (quorum sensing) and gearing for expression of other virulent factors [5]. Targeting this quorum sensing (QS)-regulated process presents a unique approach to managing bacterial infections [6]. This unconventional strategy is hypothesized to reduce the expression of virulence factors thereby disarming the pathogens without imposing evolutionary pressure [7]. Exploiting plants surviving in unique environments may possess bioactive compounds with protective mechanisms against pathogens.

Diverse medicinal plants are known to cure diseases thus gaining popularity for use due to the perception that they are safe and may act as immune boosters for the prevention or treatment of several diseases [8]. For example, Aloe ferox, has been used in the treatment of arthritis, eczema, hypertension, stress and stomach pains [9]. The leaf infusions of Brachylaena discolorare, has been used as tonics in the treatment of...
diabetes and renal conditions [10]. *Elaeodendron transvaalense* in Southern Africa is in high demand as herbal medicine often used to treat diarrhoea, stomach aches, menorrhagia, inflammations, skin infections, and rashes [11]. Many other plants are traditionally used as herbal medicine for several human diseases and ailments [12, 13, 14] thus appear as promising alternatives to traditional antibiotics [15, 16].

In this study, we examined the antibacterial, antibiofilm and anti-quorum sensing potentials of selected South African medicinal plants against diabetic foot ulcer pathogens using both in-vitro and in-silico approaches.

2. **Materials and methods**

2.1. **Plant material and solvent extraction**

Different parts of the plant species (*Aloe ferox*, *Brachylaena discolor*, *Elaeodendron transvaalense*, *Euclea natalensis*, *Euclea undulata*, *Leontis leonurus*, *Melia azedarach*, *Moringa oleifera*, *Plectranthus amboinicus*, *Sclerocarya birea*, *Solanum aculeastrum*, *Strychnos madagascariensis*, *Sutherlandia frutescens* and *Warburgia salutaris*) were collected from the University of Pretoria, Manie van der Schijff Botanical Garden, Experimental farm and Nhlelele village in Limpopo province, SA. Voucher specimens were prepared and deposited in the Herbarium of the Department of Plant and Soil Sciences, University of Pretoria, South Africa. All plant materials were dried at a room temperature (25 °C) and the plant parts (leaves, bark, and fruits) were ground or blended (IKA 2870900 MF 10.1, Cole-Parmer Scientific experts, USA) to soft powder and weighed.

Powdered material (30.0 g) per plant was extracted using 300 mL solvents of different polarities: methanol and ethyl acetate. Dichloromethane (DCM) (35.0 g) was used as it evaporates and dissolves faster. Successfully, 100 μL of MH broth was transferred in every well and 100 μL of each plant extract (in triplicate) was transferred into wells in Row A of 96-well microtitre polystyrene (PS) flat bottom clear plates (Lasec, SA) of the plate, together with the ciprofloxacin (0.01 mg/mL) (Sigma-Aldrich, SA) as the positive control and 1.0% DMSO as the negative control [20]. A blank (sterile LB broth) and standardized bacterium (control) were prepared by transferring 200 μL to the wells, respectively. Serial dilutions were prepared in the direction from A to H, resulting in decreasing concentrations (8.000–0.0625 mg/mL), thereafter the plates were incubated at 37 °C for 24 h. Approximately 40 μL of p-Iodonitrotetrazolium violet (INT) at 0.200 mg/mL (Sigma-Aldrich, SA) was added for the visual indication of bacterial growth. The appearance of the INT dye colour indicated the growth of the bacteria whereas, no colour change inferred inhibition of the bacterial growth [21]. The lowest concentration where no colour change was observed inferred the MIC value. All results were read in triplicates for statistical analysis.

2.3. **Minimum inhibitory concentrations of plant extracts**

The minimum inhibitory concentrations (MIC) of the sixty crude extracts was done following the method described in Ellof [19], with slight modification. A stock concentration of plant extracts (32.00 mg/mL) was prepared by dissolving in ~1.0% aqueous dimethyl sulfoxide (DMSO). Successfully, 100 μL of MH broth was transferred in every well and 100 μL of each plant extract (in triplicate) was transferred into wells in Row A of 96-well microtitre polystyrene (PS) flat bottom clear plates (Lasec, SA) to soft powder and weighed.

For water extraction, deionized water (300 mL) was added to cover the powdered material (30g), thereafter boiled for 45 min on a hotplate (Labotec, South Africa). After cooling, the extracts were filtered using a muslin cloth with a pore size of 2 mm. The filtrates were evaporated to dryness by a rotatory evaporator (Labotec Buchi Heidolph, Germany) at 45 °C under reduced pressure, then dried in a fume hood to complete dryness (4–14 days).

For water extraction, deionized water (300 mL) was added to cover the powdered material (30g), thereafter boiled for 45 min on a hotplate (Labotec, South Africa). After cooling, the extracts were filtered using a muslin cloth with a pore size of 2 mm, transferred to glass jars with muslin cloth with a pore size of 2 mm. The plant parts (leaves, bark, and fruits) were ground or blended (IKA 2870900 MF 10.1, Cole-Parmer Scientific experts, USA) to soft powder and weighed.

2.2. **Preparation of culture media and pathogens**

*Klebsiella pneumoniae* ATCC 33495, *Staphylococcus aureus* ATCC 25923, *Proteus mirabilis* ATCC 35583 and *Escherichia coli* ATCC 10536 were purchased from Sigma-Aldrich (South Africa) and used to evaluate the antibacterial and antibiofilm activities. *Chromobacterium violaceum* ATCC 12472, producing QS-controlled purple pigment violacein was obtained from the Centre for Microbial Ecology and Genomes (CMEG) at the University of Pretoria (South Africa) and used to evaluate the QS associated violacein production inhibition. The strains were maintained on Mueller Hinton (MH) agar (LabM; United Kingdom). *C. violaceum* was maintained in Luria Bertani (LB) (LabM; United Kingdom) agar [1% peptone, 0.5% yeast extract, 0.5% sodium chloride (NaCl), and solidified with 1.5% bacteriological agar]. All cultures were incubated at 37 °C, while *C. violaceum* was incubated at 30 °C for 24 h. For the maintenance of the bacterial strains, 50% glycerol stock cultures were prepared and kept at -80 °C until required. All cultures were standardized as per 0.5 MacFarland standards.

*2.4. Chemical profiling of active extracts using gas chromatography-mass spectrometry (GC-MS)*

The three active plant extracts chosen based on their potent inhibitory activity were subjected to GC-MS (Shimadzu QP 2100 SE: Shimadzu Corporation, Tokyo, Japan) where an Inert Cap 5 MS/NP capillary (30 m × 0.25 mm × 0.25 μm: GL Sciences, Tokyo, Japan) capillary column was used as per method in [18]. Approximately 1 μL of each sample was injected in split or split-less mode, whilst helium was used as the carrier gas (constant flow rate of 1.0 mL/min). The oven temperature was programmed at 80 °C for 3 min, increased to 280 °C at 11 °C min⁻¹, and then held at this temperature for 14 min. The analysis was carried out at 70 eV in the electron impact ionization mode. The spectra libraries NIST 11, Willey 10th edition and diagnostic ions were used for comparison of identified constituents to obtain the chemical formula, molecular weight and chemical compound names. Quantitative determinations were made by relating respective peak areas to total ion chromatogram areas from the GC-MS.

2.5. **Inhibition of QS-controlled violacein in C. violaceum**

*C. violaceum* 12472 (grown for 24 h at 30 °C on LB agar plate) was used to evaluate the inhibition of QS-controlled violacein. The standardized culture (OD₆₀₀ nm of 0.1.) was seeded evenly on LB agar plates, and sterile discs (6 mm diameter) (Lasec, South Africa) impregnated with 10 μL of crude extracts at varying concentrations (0.5, 1.0, 2.0 and 4.0 mg/mL; MIC = 8 x MIC) were fixed, incubated for 24 h at 30 °C. The positive control included cinnamaldehyde (0.50 mg/mL; Sigma-Aldrich, SA). The violacein production inhibition was observed as the presence of a creamy-white, opaque halo surrounding (diameter) the well measured in millimetres (mm) [23, 24].

Inhibition of QS-controlled violacein production in C. violaceum ATCC 12472 was quantitatively assessed according to D’Almeida et al. [25], with slight modifications using the microdilution method as described in section 2.3. The sub-MIC concentrations (0.0625–1.00 mg/mL; ½ x MIC -
2 x MIC) of plant extracts and positive control of cinnamaldehyde (0.50 mg/mL; Sigma-Aldrich, South Africa) were tested. The validation of bacteria viability was observed at 600 nm. After bacterial growth at 30 °C for 24 h with shaking at 120 rpm, the plates were subjected to oven drying at 50 °C for 24 h. Thereafter, 150 μL of 100% DMSO was used to re-dissolve the dried violaceum in each well, mixed thoroughly and placed in the shaking incubator at 30 °C, 120 rpm for 1–2 h. Thereafter, the evaluation of violacein inhibition was read at 485 nm, quantitatively. The formula below was used to calculate the percentage (%) inhibition.

\[
\text{Violacein inhibition} \ (\%) = \frac{\text{Control 485nm} - \text{Test 485nm}}{\text{Control 485nm}} \times 100
\]

\[2\]

2.6. AHL-based QS inhibition by selected compounds using molecular modelling

Compounds selected from active extracts with a chemical structure like the AHL signal molecule were subjected to molecular docking analysis as described by Perez-Lopez et al. [26] with slight modifications. Crystal structure of CviR (PDB ID: 3QP1) ligand-binding domain bound to the native ligand C6-HSL was obtained from the protein data bank. The 2-dimensional structures of the phytochemical compounds were obtained from PubChem and drawn on Canvas 3.5 and exported to Maestro 11.5.

Water (H2O) and metals were removed, prior to the optimization of the hydrogen bonds, resulting in scores that reflect the potential energy change per binding. Schrodinger’s ligprep was used to create chemically correct models of the ligands, and a protein preparation wizard was used to create the receptor structure. The grids were then docked using the Glide ligand docking module and the Glide receptor. All docking calculations were performed using AutoDock 4.0 and Grids (Schrodinger, LLC, New York, NY, USA).

2.7. Inhibitory effects of plant extracts on cell attachment

In the cell attachment inhibition assay, ~100 μL of standardized bacterial suspension (OD at 600 nm = 0.1), 100 μL of MH broth and 100 μL of extract (at MIC value) [20] were added to the wells. The positive control (0.001 mg/mL ciprofloxacin) and negative control (1% DMSO) was also added into the wells. A volume of 200 μL sterile MH broth (blank wells) was used, thereafter incubated at 37 °C for 24 h, thereafter, assessed using the modified crystal violet (CV) assay. The wells were washed three times with sterile distilled water to remove the contents. The remaining biofilm left on the walls of the wells was then oven-dried at 60 °C for 45 min. Following drying, the wells were stained with 100 μL of 1% CV solution (Merck, South Africa) as described by Ganesh and Rai [27] and incubated in the dark for 15 min. The wells were then rinsed three times with sterile distilled water to remove the excess, unabsorbed stain. To destain the wells, 125 μL of 95% ethanol was added to each well and gently swirled to dissolve the stain from the biofilm. A 100 μL portion of the destaining solution (95% ethanol) from each well was then transferred to the corresponding well of a fresh 96-well microplate and the absorbance was determined at 585 nm using a SpectraMax Paradigm microplate reader (Separations, SA). The quantitative assessment of cell attachment was performed by calculating the percentage inhibition of cell attachment by applying Eq. (3).

\[
\text{Biofilm inhibition} \ (\%) = \frac{\text{Control 585nm} - \text{Test 585nm}}{\text{Control 585nm}} \times 100
\]

\[3\]

2.8. Inhibitory effects of plant extracts on biofilm development

For pre-formed biofilm development (microcolony formation stage of biofilm development), the standardized bacterial suspension (100 μL) plus 100 μL of MH broth were added to the wells and incubated at 37 °C for 8 h. Thereafter incubation, 100 μL of extracts and 0.001 mg/mL of ciprofloxacin, positive control (Sigma-Aldrich, South Africa) were transferred into respective wells and incubated further for 24 h. Biofilm biomass was assessed using the modified crystal violet (CV) assay. The 96-well plates containing formed biofilm were washed with sterile distilled water to remove planktonic cells and media. The plates were then oven-dried at 60 °C for 45 min. Following drying, 1% CV solution (Sigma, South Africa) was used to stain the remaining biofilm for 15 min in the dark. The wells were then washed with sterile distilled water to remove any unabsorbed stain followed by adding 125 μL of 95% ethanol to destain the wells. Thereafter, 100 μL of the destaining solution was transferred to a new plate for absorbance reading at 585 nm using a multi-mode microplate reader (SpectraMax® paradigm) and determining the percentage inhibition using Eq. (3).

2.9. Selected active extracts on biofilms using confocal laser scanning microscope (CLSM)

The plant extracts that only showed the potential to inhibit biofilm development for all tested bacteria were assessed using confocal laser

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Table 1. Crude extract yields (%) attained after extraction of medicinal with solvents of different polarities.

| Plant species | Family name | Common names | Voucher specimens | Parts used | AQ | ME | DCM | EA |
|---------------|-------------|--------------|-------------------|------------|----|----|-----|----|
| Aloe ferox    | Asphodelaceae | Red aloe (E), Inlaba (Z) | 125861          | Leaf gel   | 65.5 | 58.5 | 53.0 | 61.2 |
| Brachycome discolor | Asteraceae | Coastal silver oak (E), Phabila (Z) | 125859         | Leaves     | 60.0 | 58.1 | 56.0 | 55.7 |
| Elaeodendron transvaalense | Celastraceae | Transvaal saffronwood (E), Ingwavum (Z), Mukuvhazwihi (V) | BCM 117182     | Bark       | 59.1 | 58.9 | 44.9 | 52.1 |
| Euclea natalensis | Ebenaceae | Natal guarri (E), Inkunzi emnyama (Z) | 125880         | Leaves     | 66.7 | 83.9 | 56.8 | 59.4 |
| Euclea undulata | Ebenaceae | Common guarri (E), Umgwali (Z) | 125863         | Leaves     | 65.7 | 66.2 | 56.5 | 62.4 |
| Leonotis leonurus | Lamiales | Wild dagga (E), Imunyane (Z) | 125878         | Leaves     | 65.7 | 66.2 | 56.8 | 64.3 |
| Mela azarolus | Meliaceae | Chinaberry (E), Umstilings (Z), Muserenga (V) | 125881         | Leaves     | 72.3 | 52.1 | 59.1 | 68.3 |
| Morinda oleifera | Moringaceae | Moringa, Drumstick tree | 125878         | Leaves     | 72.6 | 68.1 | 53.6 | 52.1 |
| Placopsis amboinica | Lamiaceae | Country borage (E) | 125882         | Leaves     | 58.4 | 61.6 | 48.9 | 60.8 |
| Sclerocarya birea | Anacardiaceae | Marula (E), Umnganu (Z), Mufula (V) | 125864         | Leaf, bark  | 70.9 | 66.1 | 56.7 | 62.9 |
| Solanum aculeastrum | Solanaceae | Poison apple (E), Mtuma (Z), Murulwa (V) | 125864        | Fruit      | 69.4 | 70.2 | 54.2 | 63.2 |
| Strychnos madagascariensis | Loganiaceae | Heary-leaved monkey-orange (E), Mukwakwa (V) | 125877         | Leaves     | 61.7 | 60.5 | 48.5 | 56.7 |
| Sutherlandia frutescens | Loganiaceae | Cancer bush (E), Umnozele (Z) | BCM 117163     | Leaves     | 48.5 | 65.9 | 49.4 | 51.7 |
| Warburgia salutaris | Ebenaceae | Pepperbark tree (E), Ishhaha (Z), Manaka (V) | 125860        | Leaves     | 72.4 | 58.4 | 52.9 | 61.8 |

Solvents: AQ = Aqueous; ME = Methanol; DCM = Dichloromethane and EA = Ethyl acetate. Z = Zulu, E = English, V = Venda.
scanning microscopy (CLSM) (Carl Zeiss Microscopy, Jena, Germany). Biofilm was grown on cover glass pieces placed in cell culture multi-12-well PS clear plates and incubated at 37 °C for 8 h. After 8 h of incubation, the preformed biofilm was supplemented with plant extract (1 mg/mL) or ciprofloxacin (0.001 mg/mL) (Sigma-Aldrich, South Africa), then incubated further for 24 h at 37 °C. The adherent biofilm on the cover glass pieces was gently washed three times with sterile distilled water, then stained with SYTO 9 nucleic acid and propidium iodide stain LIVE/DEAD bacterial viability kit (Invitrogen, Thermo Fisher Scientific, USA), then incubated for 15 min in the dark. The biofilms were detected using a Zeiss LSM 510 CLSM (Carl Zeiss Microscopy, Jena, Germany) by excitation at 488 nm and emission collected with a 500–530 bandpass filter [28].

### 2.10. Statistical analysis

Microsoft Excel Office (2016 version) was used to determine mean ± standard deviations for the data generated from the independent experimental repeats with each sample in triplicates. Statistical differences were assessed by the one-way analysis of variance (ANOVA) to compare the mean differences in the inhibitory activities of extracts and controls. Differences were considered at p < 0.05.

3. Results and discussion

#### 3.1. Yields from plant crude extracts

Results of the percentage yield showed methanol and aqueous as the most preeminent extractants while the least extractants were noted as ethyl acetate and dichloromethane (Table 1). The highest percentage yield was observed with methanol extract of *Euclea natalensis* (83.9%) and *Solanum aculeastrum* (70.2%), followed by aqueous extracts of *Morinda oleifera* (72.6%), *Sutherlandia frutescens* (72.4%), *Melia azedarach* (72.5%), and *Sclerocarya birrea* (70.9%).

Ethyl acetate and DCM yielded the least percentage with 51.7% from *Strychnos madagascariensis* and *Plectranthus amboinicus*, *Solanium aculeastrum* trans vaalense 48.9%, 48.5%, and 44.9%, respectively. Some solvents yield the lowest percentage than others therefore, the differences in effectiveness could be attributed to the polarity of the solvents and plant part used.

A successful determination of biologically active compound(s) from plant material is strongly dependent on the type of solvent and plant parts used in the extraction procedure [29]. Thus, selecting solvents factors, such as non-toxic to bioassay system, easy removal from extracts and safety should be considered in determining the type of solvent for extraction process [30].

#### 3.2. Validating minimum inhibitory concentrations (MIC) of the plant extracts against selected pathogens

Of the sixty (60) plant extracts tested against the four DFU associated pathogens (*E. coli*, *P. mirabilis*, *K. pneumoniae* and *S. aureus*), the results showed MIC values ranged between 0.25 mg/mL to 8.00 mg/mL (Table 2), of which thirteen (13) presented notable MIC values of ≤1 mg/mL. For *S. aureus*, a notable MIC value of 1 mg/mL was observed for *Brachylaena discolor* (ethyl-acetate), *Sclerocarya birrea* (aqueous) and *Warburgia salutaris* (methanol). *Sclerocarya birrea* (ethanol-acetate) and *Moringa oleifera* (ethanol-acetate) also showed MIC value of 1 mg/mL against *E. coli*. The MIC value was 0.5 mg/mL for *Aloe ferox* (methanol) and *W. salutaris* (aqueous). *Aloe ferox* (ethyl-acetate) and *S. birrea* (aqueous) displayed activities against *K. pneumoniae* as reflected by a significant MIC value of 1 mg/mL. Furthermore, MIC value of 1 mg/mL was measured for *B. discolor* (aqueous), *Moringa oleifera* (aqueous), *S. aculeastrum* (methanol) and *W. salutaris* (methanol and aqueous). The MIC value was 0.5 mg/mL for *Euclea natalensis* (ethyl-acetate) against *P. mirabilis*. The MIC value of 0.25 mg/mL shown by ethyl acetate extract of *Euclea natalensis* against *Proteus mirabilis* and signifies the highest activity of all plant extracts tested.

Numerous literature reports are documented regarding the antibacterial activities of the plants against several pathogens. A few of the plant extracts in the current study revealed similar activity when comparing the reports from the literature, while some displayed better than the reported activities. In a study carried out by Soeyingbe et al. [31], the antibacterial activity of *W. salutaris* aqueous extract against *E. coli*, *S. aureus* and *P. mirabilis* showed MIC values between 0.31 - 10.0 mg/mL while the methanol extracts showed MIC values that ranged between 1.25 - 5 mg/mL against *S. aureus*. This is in tandem with the results obtained from this study where an aqueous extract of *Solanum aculeastrum* also showed MIC value of 1 mg/mL against *E. coli* and methanolic extracts of *W. salutaris* revealed similar noteworthy activity (1 mg/mL) against *S. aureus*. Adamu et al. [32] also reported a MIC of 1.25 mg/mL

### Table 2. Minimum inhibitory concentration (mg/mL) of plants extracts tested against DFU associated pathogens.

| Plants Species       | K. pneumoniae | E. coli | P. mirabilis | S. aureus |
|----------------------|---------------|---------|--------------|-----------|
| Aloe ferox           | 8.00          | 8.00    | 8.00         | 8.00      |
| Brachylaena discolor | 8.00          | 8.00    | 8.00         | 8.00      |
| Solanum aculeastrum | 8.00          | 8.00    | 8.00         | 8.00      |
| Euclea natalensis    | 8.00          | 8.00    | 8.00         | 2.00      |
| Euclea undulata      | 8.00          | 8.00    | 8.00         | 2.00      |
| Leonotis leonurus    | 8.00          | 8.00    | 8.00         | 2.00      |
| Melia azedarach      | 8.00          | 8.00    | 8.00         | 2.00      |
| Moringa oleifera     | 4.00          | 8.00    | 8.00         | 2.00      |
| Plectranthus amboinicus | 8.00   | 8.00    | 8.00         | 2.00      |
| Sclerocarya birrea   | 4.00          | 8.00    | 8.00         | 2.00      |
| Sclerocarya birrea (Bark) | 8.00 | 8.00    | 8.00         | 2.00      |
| Solanum aculeastrum | 8.00          | 8.00    | 8.00         | 2.00      |
| Strychnos madagascariensis | 8.00 | 8.00    | 8.00         | 2.00      |
| Sutherlandia frutescens | 8.00  | 8.00    | 8.00         | 2.00      |
| Warburgia salutaris  | 8.00          | 8.00    | 8.00         | 2.00      |
| 1% DMSO              | 8.00          | 8.00    | 8.00         | 2.00      |
| Ciprofloxacin        | 0.0001        | 0.0001  | 0.0001       | 0.0001    |

DMSO: Dimethyl sulfoxide; 1: Dichloromethane; 2: Methanol; 3: Ethyl-acetate; 4: Aqueous.
for *B. discolor* leaves acetone extract against *S. aureus*, which is congruent to our findings, however, we used ethyl acetate. Van Vuuren [33] opined those extracts having activities where MIC values are below 8 mg/mL possess some antimicrobial activity, however, the MIC values below 1 mg/mL are considered noteworthy.

In a study carried out by Kamath et al. [34], a MIC value of 0.015 mg/mL and 0.093 mg/mL for aqueous extract of *M. oleifera* was reported against *K. pneumoniae* and methicillin-resistant *Staphylococcus aureus* (MRSA), respectively. Findings from our studies showed otherwise where an aqueous extract of *M. oleifera* revealed MIC value of 8 mg/mL for *K. pneumoniae* and 4 mg/mL for *Staphylococcus aureus*. The difference in the results observed from both studies could be attributed to the components of the plant material used, as different plants vary due to the environment and storage, the chemical composition of secondary metabolites as well as the type of bacterial species. Different strains differ in the susceptibility to antibacterial agents, thus dissimilar MIC values.

In this study, we showed the plant extracts active against both Gram-positive and Gram-negative bacteria (GNB). However, we expected the plant extracts to be more effective against Gram-positive bacteria (GPB) than GNB. This is because scientific evidence according to Delcour [35] for *A. ferox* (methanol: ME), *W. salutaris* (aqueous: AQ) and *E. natalensis* (ethyl acetate: EA) extracts. All peaks correspond to the data presented in Table 3.

![Figure 1. Representative total ion chromatograph (TIC) of *A. ferox* (methanol: ME), *W. salutaris* (aqueous: AQ) and *E. natalensis* (ethyl acetate: EA) extracts. All peaks correspond to the data presented in Table 3.](image)

### Table 3. GC-MS spectral analysis of *A. ferox* (methanol), *W. salutaris* (aqueous) and *E. natalensis* (ethyl acetate) extracts.

| Peak # | Ret. Time (min) | Name | Molecular formula | Molecular weight | *A. ferox* (ME) | *W. salutaris* (AQ) | *E. natalensis* (EA) |
|--------|-----------------|------|-------------------|------------------|----------------|--------------------|-----------------------|
| 1      | 4.46            | Decane | C₁₀H₂₂ | 142            | 6.85% | 3.86% |
| 2      | 6.28            | Pentanoic acid, 4-oxo- (CAS) | C₅H₈O₃ | 116            | 4.43% | |
| 3      | 6.47            | 4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl- | C₈H₁₀O₄ | 144            | 4.52% | |
| 3      | 6.57            | Benzenes, 2-ethyl-1,4-dimethyl- | C₁₀H₁₄ | 134            | 0.69% | |
| 4      | 9.08            | Tetradeacne | C₁₄H₁₀ | 198            | 0.35% | |
| 5      | 9.36            | Guanosine | C₁₀H₁₃N₅O₅ | 283            | 6.44% | |
| 6      | 11.62           | Trimethyl(1-methylbutoxy) silane | C₆H₁₃Si | 160            | 1.44% | |
| 7      | 11.67           | 5,6-diene-4-one | C₁₀H₁₄O | 192            | 2.44% | |
| 7      | 11.76           | Ethanol, 2,(3,3-dimethylbicycle [2.2.1] | C₁₂H₂₄O₂ | 166            | 3.67% | |
| 9      | 12.36           | Sorbitol | C₉H₁₈O₁₀ | 282            | 9.57% | |
| 10     | 12.60           | Neophytadiene | C₁₂H₁₈O₂ | 278            | 1.30% | 0.24% |
| 11     | 12.76           | Butanoic acid, 2-methyl-3,7-dimethyl-6-octenyl ester | C₁₀H₁₈O₂ | 240            | 1.10% | |
| 12     | 13.78           | 2.5,5,8a-Tetramethyl-4-methylene | C₁₅H₇O₂ | 238            | 0.92% | |
| 13     | 14.25           | α- Kaurene | C₁₅H₂₄O₂ | 272            | 0.25% | |
| 14     | 14.63           | 2.4-Heptadiene, 2,4-dimethyl | C₁₀H₁₈ | 124            | 0.98% | |
| 15     | 15.20           | Cyclohexanol, 3,3,5-trimethyl- acetate, cis- | C₁₀H₁₆O₂ | 184            | 1.01% | |
| 16     | 16.91           | 1,2-Benzene dicarboxylic acid bis-(1-ethylhexyl) ester | C₁₀H₁₈O₄ | 390            | 1.61% | |
| 17     | 17.02           | Triphenylphosphine oxide | C₁₂H₁₃OP | 278            | 1.60% | |
| 18     | 18.57           | Eicosane | C₂₀H₄₂ | 282            | 0.66% | |
| 19     | 18.93           | Squalene | C₂₀H₄₀ | 410            | 0.37% | |
| 20     | 25.58           | α-Amyrin | C₂₀H₃₂O₂ | 426            | 4.30% | |
| 21     | 20.61           | Hexacosane | C₂₀H₴₀ | 366            | 0.54% | |
| 22     | 20.62           | Octadecone, 1-iodo- | C₁₈H₃₂I | 380            | 1.08% | |
| 23     | 24.59           | Vitamin E | C₂₀H₃₂O₂ | 430            | 1.17% | |
| 24     | 25.72           | Doctrinacontane | C₂₂H₄₆O | 450            | 1.64% | |
| 25     | 25.73           | Docosane | C₂₂H₄₆O | 310            | 5.79% |
indicate that GNB possesses a protective outer membrane (OM), which makes it less permeable to antimicrobial compounds. In this case, the average antibacterial activities against the GNB were slightly better than the activities against the GPB. This suggests the extracts to possess ability to penetrate OM of GNB.

Based on the solvent polarity, methanol and aqueous extractants play a role in the biological activity, revealing that these extracts and associated compounds may offer prospective treatment of infections. The least antibacterial activities obtained from the ethyl acetate and DCM extracts cannot be concluded. This may be due to the inadequate quantities of active constituent(s) in the extract, exhibiting the observed antibacterial activity.

3.3. Gas chromatography-mass spectrometry (GC-MS) analysis

The utilization of GC-MS analysis was effective in identifying the bioactive compounds of *A. ferox* (methanol), *W. salutaris* (aqueous) and *E. natalensis* (ethyl-acetate) (Figure 1). These plants extracts were chosen based on their active or potent antibacterial activities and to limit the scope of this paper. Table 3 shows retention time, molecular formula, molecular weight and concentration (peak area %) of the identified compounds.

About 25 bioactive phytochemicals were identified while 7 of the compounds were Pentanoic acid, 4-oxo-(CAS), 4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-, Guanosine, 1,2-Benzenedicarboxylic acid bis-(1-Ethylhexyl) ester and Triphenylphosphine oxide were identified in *A. ferox* (methanol). Eleven compounds were Trimethyl(1-methylbutoxy) silane, Cis Megastigma-5,8-diene-4-one, Ethanol, 2-(3,3-dimethylbicyclo hept-2-ylidene)-, Sorbitol, Neophytadiene, Butanoic acid, 2-methyl-, 3,7-dimethyl-6-octenyl ester, 2,5,5,8a-Tetramethyl-4-methylene, 2,4-Heptadiene, 2,4-dimethyl-, Cyclohexanol, 3,3,5-trimethyl-, acetate, cis-Octadecane, 1-ido- and Docosane identified from *W. salutaris*. Ten (10) compounds identified from *Eucalyptus natalensis* (ethyl-acetate) are Benzene, 2-ethyl-1,4-dimethyl-, Tetradecane, Neophytadiene, α-Kaurene, Eicosane, Squalene, Hexacosane, Vitamin E, Drotiacontane and α-Amyrin. The mass spectra of the current results was compared with mass spectra constituents of the NIST (national library of standards and technology) library.

3.4. Antagonistic effect of compounds against AHL-based QS of *Chromobacterium violaceum* (AQS)

Few attempts have been made to test AQS potential activities of most medicinal plants and herbs. Therefore, more studies to investigate the AQS activity should be considered. The significance of this novel strategy offers anti-infective therapy, as it intentionally delays the expression of pathogenic traits, rather than blocking the pathogen’s growth [36].

Based on the qualitative evaluation of AQS activity of the plant extracts, there was no interference on violacein (purple pigment) production in *Chromobacterium violaceum*. Alternately, bactericidal effect of plant extract on the biomonitor strain was observed. Since *Cosa et al.* [37] reported similar findings and suggested that most of the plant extracts rather display AQS activity when evaluated quantitatively, thus, quantitative assays should be considered and optimized for AQS screening and in a similar manner employed in MIC determination. The quantitative method allows for avoidance of discrepancies between two widely used methods. For this reason, we, proceeded to evaluate the plant extracts using a quantitative AQS assay.

The results (Figure 2) obtained from this study revealed that some plant extracts at low concentrations were enhancing the growth of the bacterium, however showed inhibitory effect at higher concentrations. These extracts yielded a high percentage inhibition against violacein production at dose-dependent concentration. The violacein inhibition of the active extracts of *W. salutaris* (aqueous) 14.97–46.89%, *E. natalensis* (ethyl-acetate) 20.09–58.34% and *A. ferox* (methanol) 4.81–54.83% at increasing concentrations (*V*/&nbsp;MIC - 2 x MIC in mg/mL). *E. natalensis* ethyl-acetate yielded the best results across all the concentrations tested. The positive control cinnamaldehyde exhibited 69.05–74.95% violacein inhibition.

The results obtained in this study corresponds to the inhibition values from literature research for other medicinal plants and herbs. For example, Baloyi *et al.* [22], reported the inhibition between 57.55% - 71.65% for the studied plant extracts. *Cosa et al.* [37] also reported that 11 out of 56 extracts inhibiting violacein production (90%) when applied at higher concentration (7.00 mg/mL). In a study by *Vattem et al.* [38], it was reported that basil had the highest activity with 78%, thyme and brassica oleracea had 60% they were followed by rosemary, ginger, and turmeric which decreased violacein formation by 40%. Inhibition of violacein production was observed in certain studies, this might be due to the plant compound’s activity during the interaction of AHL with the signal receptor [39]. We therefore can deduce that medicinal plants and phytochemicals inhibit QS by blocking the production of AHL signal molecules. The use of inducer analogues, inactivation of the inducer receptors, inhibition of the downstream effects of QS and enzymatic inactivation of QS signals [40]. Additional research is needed to further understand the mechanisms that induce plant based QSI.
### 3.5. Molecular modelling of phytochemicals as antagonists of AHL-based QS

Molecular docking plays a role in predicting the intermolecular framework developed and understanding the drug-receptor interactions, and it is suggested that the attachment modes are accountable for inhibition of the protein [41]. It allows for prediction and binding orientation of small molecule drug candidates to their protein targets. A notable docking score is between -5 to -15 kcal/mol [42]. Identified phytochemicals of 3 active extracts were examined for their structure-activity relationships with AHL receptor protein homologues CviR (PDB: 3QP1). The LuxR receptor protein found in C. violaceum ATCC 12472 is the CviR (PDB: 3QP1) and it is activated by its cognate ligand, 3-hydroxy-C10-AHL, and responds to C10-AHL while C6-AHL acts as a fractional antagonist. CviR shares 87% amino acids sequence identity to CviR [43] and therefore the interactions vary. Table 4 highlights the docking energy, docking score, glide energy and interaction associated with the most favourable docking pose for each ligand complexed with the 3QP1 (C. violaceum ATCC 12472) protein.

From the docking results, about 14 out of the 25 compounds could bind the CviR protein. Sorbitol showed the highest docking score of -7.049 kcal/mol, followed by guanosine (-6.634 kcal/mol) and 1,2-Benzenedicarboxylic acid (-6.225 kcal/mol). These scores can be slightly compared to quercetin (the positive control) which showed a docking score of -10.611 kcal/mol. The least docking score was observed in tetradecane (-2.820 kcal/mol). This suggests that the compounds have high stability. More negative binding free energy values represent tighter binders [44]. Supportive energy for the protein-ligand binding interaction is described by high negative glide energy. These results, therefore, suggest that the compounds can serve as better antagonists, therefore, promoting the open conformation of CviR protein.

The docked complex visualization showed that hydrophobic forces and hydrogen bonding play a significant role in their host-guest relationship. The molecular docking interaction network between N-acyl-L-homoserine lactone (AHL) and the studied compounds are shown in Figure 3.

A lactone head can form an H-bond with the adjacent Trp84 residue, the acyl group forms H-bonds with Leu100, Ile99 and Asp97. Bodede et al [45] reported that the acyl group forms H-bonds with Asp97, Tyr80, and Ser155. The tail part is buried in a hydrophobic pocket made of Val, Leu, and Ile residues [46]. Based on molecular modelling studies, ligands acting as AHL antagonists usually possess a hydrophobic tail which facilitates binding of these ligands with the active site by H-bonding and hydrophobic interactions [47].

### 3.6. Biofilm inhibition formation of initial cell attachment

Cell attachment is the first stage of biofilm formation crucial as the cells easily attach to a surface if left untreated, thus forming matured biofilm thus appears appealing in wound healing.

![Figure 3. Molecular docking interaction of compounds with good binding scores, Tetradecane (compound with the least binding score) and positive control. A: Guanosine; B: 1,2-Benzenedicarboxylic acid; C: 2,3-Dihydro-3,5-dihydroxy-6-methyl-4h-pyran-4-one; D: 2-(3,3-Dimethylbicyclo(2.2.1) hept-2-ylidene) ethanol; (E): 5,8-Megastigmadien-4-one; F: Sorbitol; G: 1,4-Dimethyl-2-ethylbenzene; H: Tetradecane; I: Quercetin; J: N-acyl-L-homoserine lactone. The negatively charged residues are shown in red, polar residues in blue, and hydrophobic residues in parrot green. Hydrogen bond (H-bond) interactions are shown as a pink arrow.](image-url)
Warburgia salutaris aqueous, A. ferox methanol and E. natalensis ethyl acetate plant extracts blocked the cell attachment by 73.86%, 38.49%, and 25.90% against E. coli, respectively (Figure 4). Low levels of inhibition were observed for three extracts, W. salutaris aqueous, A. ferox methanol and E. natalensis ethyl acetate against S. aureus, by 30.26%, 23.29% and 22.49%, respectively. Similar results were obtained against K. pneumoniae. The least percentage inhibition was exhibited for P. mirabilis using W. salutaris aqueous, A. ferox methanol and E. natalensis ethyl acetate (8.26%, 9.90% and 3.70%), respectively. Ciprofloxacin (0.001 mg/mL), the positive control inhibited the bacterial cell attachment by 52%–88% for all pathogens (Figure 4).

The results indicated the ability of the extracts to inhibit bacterial cell attachment to the surface of the microplate by 73%. For this reason, W. salutaris aqueous extract (73%) against E. coli, displayed good activity, the outcome of this assay recommend that all these three extracts may possess QS compounds that may be beneficial to avert the aggregation of pathogenic bacteria in the early stages of infection and therefore delay/deter biofilm formation.

Reports on the antibiofilm activity study of plant extracts are limited. Omwenga et al. [7] reported Warburgia ugandensis Sprague almost completely inhibited the E. coli at concentrations of 1 mg/mL and 0.5 mg/mL. Biofilm formation inhibition results of plant extracts against E. coli and S. aureus indicated that the obtained effect was concentration-dependent, as the study was done in a sub-inhibitory concentration (75, 50, and 25%) the best biofilm reduction is observed in higher concentrations of the extracts [49], however, on the current study the assay was only done using one concentration (1 mg/mL).

3.7. Effect of plant extracts on preformed biofilm

According to Baloyi et al. [22], when bacteria are attached to an appropriate surface, the biofilm starts to accumulate biomass as it develops. The results (Figure 5) displayed different effects as compared to the cell attachment. Weak reduction or no inhibition, for both the plant extracts and positive control (ciprofloxacin) was observed. Two extracts, A. ferox methanol, and E. natalensis ethyl acetate inhibited the preformed E. coli biofilm by 15.00% and 17.44% respectively and inhibited the preformed K. pneumoniae biofilm by 0.92% and 12.57% respectively, while none of the plant extracts were able to destroy the preformed S. aureus and P. mirabilis biofilms (Figure 5). Although some of the plant

Figure 4. Effect of plant extracts on biofilm adhesion of test bacterial pathogens (P. mirabilis, E. coli, S. aureus and K. pneumoniae). Data is represented as the percentage inhibition of biofilm formation. Comparison of percentage inhibition at 1 mg/mL for each extract against tested bacteria with different letters (a–f) are significantly different (p ≤ 0.05). ME: methanol; EA: ethyl-acetate; AQ: aqueous.

Figure 5. Effect of plant extracts on preformed biofilm of test bacterial pathogens S. aureus, P. mirabilis, E. coli, and K. pneumoniae. Data is represented as the percentage inhibition of biofilm formation. Comparison of percentage inhibition at 1 mg/mL for each extract against tested bacteria with different letters (a–f) are significantly different (p ≤ 0.05). ME: methanol; EA: ethyl-acetate; AQ: aqueous.

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extracts showed the ability to inhibit the development of the preformed biofilms, they also displayed weak activity as the percentage inhibition was ≤30%. The positive controls displayed a decrease in percentage inhibition, but they seem to be more active as compared to the plant extracts against all test pathogens.

Eradicating pre-existing biofilms by plant extracts is more challenging. From this phenomenon, it can be validated that pathogens can resist the mode of combating antimicrobials more when they exist in a community and their infections are capable to preserve on different biotic and abiotic surfaces [50]. Some of the basis of resistance in biofilms involve the presence of an extracellular polymeric matrix (EPS) which instigates strong attachment of microbes to surfaces and little antibiotic penetration or increased activity of efflux pumps which eject antimicrobial agents from cells [51]. The enhancement of biofilm production by some of the extracts confirm initial reports that some natural compounds may promote the growth of microbes [21]. A possible explanation is that some of the metabolites present in the plant extracts were used as a source of nutrition by the bacteria.

3.8. Microscopic biofilm inhibition visualization

To further validate the antibiofilm potential of plant extracts, microscopic images of E. coli, K. pneumoniae, P. mirabilis, and S. aureus biofilms (both treated and untreated) were observed using a confocal laser scanning microscope (CLSM) (Figure 6). The mixture dyed the bacteria to reveal the disrupted cell to appear as either yellow or red, while the live viable cells appear green (Figure 6.4).

The images confirmed that the plant extracts contributed to formed dense biofilm (Figure 6.4A & Figure 6.4B). The positive controls reduced the viable cells, as a slight reduction was observed for K. pneumoniae and E. coli. The plant extracts A. ferox (methanol) and E. natalensis (ethyl-acetate) to slightly contribute to the aggregation of cells. W. salutaris (aqueous) extract reduced the number of cells, but there was no evidence of disintegration nor disruption of cells (Figure 6.4C & Figure 6.4D).

Images of the result (Figure 6) validate the weak inhibitory effect of the biofilm development obtained for the plant extracts against the tested pathogens and an enhancement of the biofilm is observed.

4. Conclusions

The present study showcased the potentials of selected medicinal plants as antibacterial, AQS and antibiofilm agents, against DFU associated pathogens. Molecular modelling confirmed 8 compounds which bound to the CviR’ protein by mimicking the AHL molecule, which is produced by Gram negative bacteria, however, in the case of Gram-positive bacteria, the autoinducing peptides will be targeted. A. ferox, E. natalensis and W. salutaris interfered with bacterial cell attachment. To the best of our knowledge, this is the first report of A. ferox, E. natalensis and W. salutaris plants with the AQS and antibiofilm activity. These plants and their respective compounds may be further explored as suitable candidates for the treatment of DFU and the development of potential phytomedicines.

Declarations

Author contribution statement

Tebats G. Mashamba: Conceived and designed the experiments; Performed the experiments; Wrote the paper.

Idowu J. Adeosuna: Analyzed and interpreted the data; Wrote the paper.

Itumeleng T. Baloyia: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.
Emmanuel T. Tshikalange: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.
Sekelwa Cosam: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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The authors declare no conflict of interest.

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