

Full Length Research Paper

Antibacterial, antiquorum sensing, antibiofilm activities and chemical profiling of selected South African medicinal plants against multi-drug resistant bacteria

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South African indigenous plants have been predominantly studied for their antibacterial abilities, overlooking their antipathogenic and antivirulence (also known as antiquorum sensing) potential. Hence, this study explored the selected medicinal plants as possible agents capable of interfering with bacterial growth, quorum sensing and biofilm formation and identified their respective bioactive compounds. Ten medicinal plants were extracted with varied solvents. *Melianthus comosus* (dichloromethane, aqueous and methanol), *Pelargonium sidoides* (aqueous) and *Vachellia karroo* (aqueous and methanol) extracts showed potent minimum inhibitory concentration values ranging from 0.19 to 0.78 mg/ml against the tested bacterial pathogens: *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Streptococcus pyogenes*, and *Escherichia coli*. Reduced violacein production (up to 38.34%) in *Chromobacterium violaceum* was noted for *Melianthus comosus*, *Plectranthus ecklonii* and *Pelargonium sidoides* extracts. Treatment of five MDR bacterial pathogens with active extracts for anti-adhesion and biofilm development showed up to 66.34 and 31.82% inhibitory effects, respectively. Chemical characterization of active extracts revealed compounds such as α -D-glucopyranoside, methyl, linalool, octadecanoic acid and hexadecanoic acid. The biological assays validated the tested plant extracts as having antibacterial and antipathogenic potentials that could be used against multidrug resistant bacteria.

Key words: Antibacterial activity, biofilm inhibition, chemical characterization, multidrug resistance, phytochemicals, quorum sensing inhibition.

INTRODUCTION

Multidrug-resistant (MDR) pathogens progressively develop within the public and health care system, creating

a menace worldwide (Karunanidhi et al., 2018). The most prevalent infections such as pneumonia, bacteraemia,

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intra-abdominal, urinary tract, skin and soft tissue infections are caused by pathogens such as *Escherichia coli*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Streptococcus pyogenes* and *Pseudomonas aeruginosa* which are associated with multidrug resistance (Bhatia et al., 2021). Amidst bacterial pathogens, MDR is usually caused by a build-up of genes with every coding developing resistance to a single drug on R plasmids (Neuenschwander, 2009).

The increased rate of MDR bacteria is elicited due to the bacterial proficiency in efflux pump use, expelling diverse, structurally unrelated compounds from the cell, conferring resistance to an extensive choice of antibiotics (Tang et al., 2014). These MDR bacteria are also known to develop biofilms and contribute towards bacterial pathogenicity by triggering genes that regulate their virulence thus reducing the potency of antibiotics (Guzzo et al., 2020). The formation of biofilms through bacterial communication termed quorum sensing (QS), a threshold of signaling molecules in high population density, leads to the regulation of processing specific genes (Sharma et al., 2019). Pathogenic bacteria exhibit resistance to many antibiotic classes; hence, the discovery of novel antipathogenic drugs or plant-based antibiotics other than those isolated from microbes continues (Munita and Arias, 2016).

Furthermore, the traditional strategy of antibiotic discovery has reduced, leading us to a world with ineffective antibiotics (Mulani et al., 2019). For many years, plant-based remedies are vastly produced from crude plant extracts that contain various mixtures of secondary metabolites (Konappa et al., 2020). This has enthused the development of novel drugs from secondary metabolites or naturally derived compounds, as they have become the basis for the enhancement of medicine or therapeutic compounds used for treating diseases (Konappa et al., 2020).

Thus, the emergence of microbial resistance to vast classes of antibacterial agents necessitates focusing on QS regulatory systems as a possible alternative approach to managing drug resistance (Zhao et al., 2020). To date, the control of bacterial infections through the QS disruption mechanism may be necessary by (i) decreasing the production of, (ii) or destroying signal proteins (autoinducers), otherwise by (iii) competitively binding acyl-homoserine lactone (AHL) mimickers to the receptor protein (Moradi et al., 2020). There are possibilities that medicinal plants could possess or use other mechanisms of action such as the antiquorum sensing strategy to impede pathogenic bacteria (Bouyahya et al., 2017).

Therefore, South African medicinal plants that may possess bioactives with the potential to disrupt QS signaling through either of the proposed mechanisms were sought. South African traditional plants are well documented mostly for antibacterial activity, whereas the associated antipathogenic properties are limited.

The rationale of the study was to validate the antibacterial activities of ten chosen South African medicinal plants against MDR pathogens, assess their antipathogenic potential, prohibit or disrupt biofilm formation and perform chemical characterization analysis of bioactive plant extracts.

MATERIALS AND METHODS

Chemicals, media and reagents

Acetone, ciprofloxacin, cinnamaldehyde, crystal violet, dimethyl sulfoxide (DMSO), dichloromethane, ethyl acetate, methanol and N, O-bis(trimethylsilyl) trifluoroacetamide (BSTFA) were procured from Sigma (Johannesburg, South Africa). Media of Luria Bertani and Muller Hinton were obtained from Lasec (Johannesburg, South Africa).

Plant collection, identification and extraction process

Ten medicinal plants (Table 1) were harvested from the Manie van der Schijff botanical garden in February 2019. Leaves of the plants were authenticated by Ms Magda Nel and voucher specimens were placed at the H.G.W.J. Schweickerdt herbarium. The leaves were thoroughly washed and air-dried at an ambient temperature for 3 to 7 days. Subsequently, the desiccated leaves for each plant sample were pulverized into fine powder by using a cutting grinder. The plants were extracted using maceration (organic solvents) and decoction (aqueous) techniques (Khan et al., 2018). The fine powder of plant materials (25g) was macerated with 250 ml of acetone, ethyl acetate, dichloromethane and methanol with shaking at 150 rpm for 48 h. The extracts were sieved with Whatman filter paper (No.1) dried by rotary evaporator at 50°C and kept at 4°C until further analysis. The aqueous extracts were prepared as a decoction by soaking 25 g of fine powdered plant material in 250 ml of sterile distilled water, allowed to simmer for 15 min and then cooled off. The filtrated samples were then placed in glass bottles and freeze-dried. The dried crude extracts were weighed and kept in glass vials (10 ml). A 1% aqueous dimethyl sulfoxide (DMSO) was used to redissolve the 50 crude extracts by sonication then prepared to the required concentrations. The extraction yields (%) for all extracts were determined by applying Equation 1.

$$\text{Extraction yield (\%)} = (\text{Dry plant extract}) / (\text{Dry initial plant material before extraction}) \times 100 \quad (1)$$

Bacterial strains

The bacterial strains: *Escherichia coli* ATCC 10536, *Klebsiella pneumoniae* ATCC 33495, *Pseudomonas aeruginosa* ATCC 9721, *Staphylococcus aureus* ATCC 25923, and *Streptococcus pyogenes* ATCC 19615 were acquired from Sigma-Aldrich (Johannesburg, South Africa). *Chromobacterium violaceum* ATCC 12472 (biomonitor strain) was used for the qualitative and quantitative evaluation of AQS, which was kindly provided by the Centre for Microbial, Ecology and Genomics (CMEG), University of Pretoria, South Africa. Ethics approval for the use of these microorganisms was obtained from the University of Pretoria, Faculty of Natural and Agricultural Sciences Ethics Committee (reference number: NAS158/2021).

The Clinical and Laboratory Standard Institute guidelines were followed for these bacterial growth media and conditions (Clinical and Laboratory Standards Institute, 2020). Bacterial cultures of *E.*

Table 1. South African medicinal plants associated with treating nosocomial infections.

Plant species	Family name	Common name	Plant parts used	Voucher specimen	References
<i>Acokanthera oppositifolia</i> (Lam)	Apocynaceae	Inhlungunyembe (Z)	Leaves	PRU 125454	Adedapo et al. (2008)
<i>Aloe ferox</i> (Mill)	Aloaceae	Khala (Z)	Leaves	PRU 125455	Chen et al. (2012)
<i>Artemisia afra</i> (Jacq. ex Willd)	Asteraceae	Mhlonyane (Z)	Leaves	PRU 125456	Liu et al. (2009)
<i>Lippia javanica</i> (Burm.f. Spreng)	Verbanaceae	Lemon bush (E)	Leaves	PRU 125882	Maroyi (2017b)
<i>Melianthus comosus</i> (Vahl)	Meliantaceae	Honey flower (E)	Leaves	PRU 125457	Eloff et al. (2018)
<i>Pelargonium sidoides</i> (DC)	Geraniaceae	Ikubalo (Xh)	Leaves	PRU 125883	Kumar et al. (2015)
<i>Plectranthus ecklonii</i> (Benth)	Lamiaceae	Tall spurflower (E)	Leaves	PRU 125458	Nyila et al. (2010)
<i>Tetradenia riparia</i> ((Hochst.) Codd)	Lamiaceae	Ibosa (Z)	Leaves	PRU 125459	Coopoosamy and Naidoo (2011)
<i>Trichilia emetica</i> (Vahl)	Meliaceae	Umathunzi (Z)	Leaves	PRU 125460	Komane et al. (2011)
<i>Vachellia karroo</i> (Hayne. Banfi and Glasso)	Fabaceae	Umunga (Z)	Leaves	PRU 125884	Maroyi (2017a)

Z: Zulu; Xh: Xhosa; E: English.

coli, *K. pneumoniae*, *P. aeruginosa*, *S. aureus* and *S. pyogenes* were prepared in Mueller Hinton (MH) medium and incubated at 37°C while *C. violaceum* was prepared in Luria Bertani (LB) medium and incubated at 30°C. A 0.5 Mc Farland standard equivalent was achieved by transferring a single or two colonies to sterile distilled water to reach OD_{600nm} = 0.1.

Microdilution assay

The fifty crude extracts were evaluated for minimum inhibitory concentrations (MICs) against *E. coli*, *K. pneumoniae*, *P. aeruginosa*, *S. aureus* and *S. pyogenes* using the microdilution method, with slight modifications (Pauw and Eloff, 2014). A volume of 100 µl (Mueller-Hinton broth, MHB) was added to each well in a microtiter plate. In the first-row wells, 100 µl of each plant extract (in triplicate), including 100 µl of positive (ciprofloxacin, 0.01 mg/ml) and negative (1% dimethyl sulfoxide) controls were transferred into respective wells. Thereafter, 200 µl of sterile MH broth which served as blank was also transferred to the wells. A series of 2-fold serial dilutions were performed (6.25 - 0.048 mg/ml) then standardised microbial suspension (100 µl) was transferred to the wells, incubated at 37°C for 24 h. Then the addition of 0.2 mg/ml iodinitotetrazolium (40 µl) into the wells and

incubated further for 1 h. The minimal inhibitory value of the extract against bacterial growth was assessed visually and recorded.

Influence of crude extracts on violacein production

Comparatively, of the two methods (qualitative and quantitative violacein assays) used to evaluate the QS inhibitory effects of the extracts, the modified microdilution assay is more appropriate in contrast with disc diffusion assay. This may be attributed to the secondary metabolites in the plant extracts (Yüzbaşıoğlu et al., 2017).

Qualitative violacein production inhibition assay

The detection of AQS activity of the crude extracts was carried out using the disc diffusion assay, following the method described by Chenia (2013) with slight modification. The standardised microbial suspension was swabbed over the entire surface of the agar plate. Thereafter, a 10 µl aliquot was loaded on sterile discs (6 mm diameter) with different concentration of each plant extracts (6.25, 3.12, 1.56 and 0.78 mg/ml) and positive control of cinnamaldehyde (0.50, 0.25, 0.12 and 0.06 mg/ml), allowed to air-dry, then incubated for 24 h at 30°C. The assessment

of violacein production was indicated with the appearance of an opaque zone (AQS activity) and clear zones (bactericidal activity) encircled around the discs. The pigment zones inhibition were determined as described by Zahin et al. (2010).

Quantitative violacein production inhibition assay

The microdilution method was used to quantitatively determine the violacein reduction of plant extracts and positive control of cinnamaldehyde against *C. violaceum* ATCC 12472 (Almeida et al., 2017). The viability and bacterial growth were checked at an absorbance of OD_{600 nm} while for violacein production at an absorbance of OD_{485 nm} and incubated at 30°C with agitation at 120 rpm for 24 h. After incubation, absorbance was read again at OD_{600 nm}. The plates were dried in an oven at 50°C for 24 h. Subsequently, 150 µl of 100% DMSO was resuspended in each well, blended with the dried samples and placed in a shaker at 120 rpm, 30°C for 1 to 2 h. Then, for quantification of violacein, the absorbance was read at OD_{485 nm}. The percentage inhibitory value was assessed by applying Equation 2:

$$\text{Percentage inhibition} = (\text{OD control} - \text{OD test}) / (\text{OD control}) \times 100 \quad (2)$$

Inhibitory effects of plant extracts on cell attachment and biofilm development

The antibiofilm activity method was followed as described by Famuyide et al. (2019), with slight modifications. Pathogens of *S. aureus*, *S. pyogenes*, *E. coli*, *P. aeruginosa* and *K. pneumoniae* were assessed for biofilm growth inhibition using six plant extracts of *M. comosus* (dichloromethane, aqueous and methanol), *P. sidoides* (aqueous) and *V. karroo* (aqueous and methanol) with significant MIC values.

For cell attachment assessment, the following equivalent volumes of 100 µl were added to the respective wells: MH broth, followed by each extract then ciprofloxacin (0.001 mg/ml) and 1% DMSO as a positive and negative control, respectively. Subsequently, 100 µl of microbial culture suspension of 0.1 at OD_{600 nm} was also added. An aliquot of 200 µl (sterile MH broth) was filled for blank wells and placed in an incubator at 37°C for 24 h. Similarly, for biofilm development assessment, the same procedure mentioned earlier was applied, except only 100 µl aliquots of microbial culture suspension and MH broth were added and incubated at 37°C for 8 h. After 8 h incubation, equal volumes of 100 µl of extracts and controls were then added to the specific wells and incubated further for 24 h.

The crystal violet (CV) method was then applied to evaluate biofilm inhibition. The removal of any free-floating cells and media from the 96-well plates were rinsed with sterile distilled water. The plates were then oven-dried at 60°C for 45 min. After drying, for 15 min in the dark, 1% CV solution was used to dye the residual biofilm. To eliminate any unabsorbed dye, the wells were rinsed with sterile distilled water. Thereafter, 125 µl of 95% ethanol was added to destain the wells. A 100 µl portion was removed into another clean plate and determined the absorbance at OD_{585 nm} using a microplate reader. The determination of percentage inhibition was done applying Equation 2. The following classification for the analysis of results was used: good activity represented by ≥ 50%, weak activity within the range of 0 and 49% whereas negative values represented growth enhancement rather than biofilm inhibition (Famuyide et al., 2019).

Gas chromatography-mass spectrophotometry (GC-MS) analysis

Plant extracts showing noticeable inhibition were analysed by GC-MS with direct injection or after silylation as trimethylsilyl derivatives of the nonvolatile components. The trimethylsilyl derivatives were acquired by adding 10 µl of N,O-bis(trimethylsilyl) trifluoroacetamide (BSTFA) and 10 µl of the plant samples into the GC-MS vials. For derivatization to take place, the mixture was left overnight at a -20°C. Chemical characterization of the extracts was conducted on a Shimadzu QP 2100 SE outfitted with an InertCap 5MS/NP capillary (30 m × 0.25 mm × 0.25 µm) capillary column. Depending on the concentration of the extracts, 1 µl of each sample was added to the GC-MS in split or split-less mode. The carrier gas (Helium) was used at a constant flow rate of 1.0 ml/min. The following oven temperature parameters were fixed at 80°C for 3 min, increasing to 280°C at 11°C min⁻¹, and held for 14 min. The analysis was carried out at 70 eV in the electron impact ionisation mode. Detection of compounds and comparison with available mass spectra libraries and diagnostic ions was made based on their similarities.

Statistical analysis

Data were expressed as the mean ± standard deviation for each sample, and treatments were conducted in triplicate. The statistical analysis system (SAS) program was used for all statistical analyses

and the statistical significance of p<0.05 value was considered.

RESULTS

Plant collection and extraction yields

Maceration and decoction extraction were performed yielding various crude extracts reported in Table 2. Quantitatively, aqueous and methanolic extracts gave greater yields than ethyl acetate, acetone and dichloromethane (Table 2). Methanolic solvent had the highest yields from *Pelargonium sidoides* (35.9%), *Acokathera oppositifolia* (27.1%) and *Artemesia afra* (25.7%), while aqueous solvent extracted the highest yields from *Aloe arborescens* (31.5%), *Artemesia afra* (24.3%) and *Melianthus comosus* (21.1%). *Aloe arborescens* showed the lowest yields of 2.5, 1.9 and 1.4% for acetone, dichloromethane and ethyl acetate, respectively.

Minimum inhibitory concentrations (MICs)

The antibacterial activities of the studied plant extracts (50) against MDR pathogens (*P. aeruginosa*, *S. pyogenes*, *E. coli*, *S. aureus* and *K. pneumoniae*,) are presented as MICs (mg/ml) in Table 3, indicative of noteworthy or insignificant activities. The MIC values ranged from 0.19 to 6.25 mg/ml. *Artemesia afra* (acetone and ethyl acetate), *Melianthus comosus* (aqueous, methanol, acetone and dichloromethane), *Tetradenia riparia* (acetone), *Plectranthus ecklonii* (methanol and dichloromethane), *Pelargonium sidoides* (aqueous, methanol and dichloromethane) and *Vachellia karroo* (aqueous, methanol and acetone) showed promising activities against the tested Gram-negative and Gram-positive bacteria (Table 3).

As anticipated, Gram-positive bacteria (GPB), *S. aureus* and *S. pyogenes* were the most vulnerable to the crude extracts; thus, potent activities were observed. Methanol extract of *M. comosus* had a MIC value of 0.19 mg/ml, aqueous and dichloromethane *M. comosus* extracts both showed MIC of 0.78 mg/ml showed significant activity while *A. afra* (acetone and ethyl acetate), *M. comosus* (acetone) and *V. karroo* (aqueous) extracts showed MIC value of 1.56 mg/ml against *S. aureus*. *M. comosus* (methanol) extract exhibited a MIC value of 0.19 mg/ml against *S. pyogenes*. Aqueous and dichloromethane *M. comosus* extracts had MICs of 0.39 mg/ml. *P. sidoides* (aqueous) extract and *V. karroo* (aqueous and methanol) extract showed the same MIC value of 0.78 mg/ml against *S. pyogenes*. The seven extracts of *M. comosus* (aqueous), *P. ecklonii* (acetone and dichloromethane), *P. sidoides* (methanol, dichloromethane), *T. riparia* (acetone) and *V. karroo* (acetone) all showed a MIC value of 1.56 mg/ml against *S. pyogenes*. For the three GNB tested, only

Table 2. Crude plant extract yields (% w/w) obtained after extraction of medicinal plants with solvents of varying polarities.

Medicinal plant	Plant part	Solvents (%)				
		Aqueous	Methanol	Acetone	Ethyl acetate	Dichloro-methane
<i>Acokathera oppositifolia</i>	Leaves	16.2	27.1	11.5	7.2	14.9
<i>Aloe arborescens</i>	Leaves	31.5	7.2	2.5	1.4	1.90
<i>Artemesia afra</i>	Leaves	24.3	25.7	9.2	9.5	23.4
<i>Lippia javanica</i>	Leaves	18.9	7.8	4.9	3.6	8.0
<i>Melianthus comosus</i>	Leaves	21.1	22.1	6.4	3.6	2.2
<i>Pelargonium sidoides</i>	Leaves	18.8	35.9	5.2	2.9	3.6
<i>Plectranthus ecklonii</i>	Leaves	18.0	10.4	6.4	6.3	2.6
<i>Tetradenia riparia</i>	Leaves	17.5	6.7	4.9	4.1	3.5
<i>Trichilia emetica</i>	Leaves	18.9	20.0	3.6	3.9	5.0
<i>Vachellia karroo</i>	Leaves	9.2	12.3	7.3	2.4	4.7

Table 3. Minimum inhibitory concentrations (mg/ml) of plant extracts against *S. pyogenes*, *K. pneumoniae*, *P. aeruginosa*, *E. coli* and *S. aureus*.

Plant extracts	<i>E. coli</i> ATCC 10536					<i>K. pneumoniae</i> ATCC 33495					<i>P. aeruginosa</i> ATCC 9721					<i>S. aureus</i> ATCC 25923					<i>S. pyogenes</i> ATCC 19615				
	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
<i>Acokathera oppositifolia</i>	6.25	6.25	6.25	6.25	6.25	6.25	6.25	6.25	6.25	6.25	6.25	6.25	6.25	6.25	6.25	6.25	6.25	3.12	6.25	6.25	6.25	6.25	6.25	6.25	6.25
<i>Aloe arborescens</i>	6.25	6.25	6.25	6.25	6.25	6.25	6.25	6.25	6.25	6.25	6.25	6.25	6.25	6.25	6.25	6.25	6.25	6.25	3.12	6.25	6.25	3.12	3.12	3.12	3.12
<i>Artemesia afra</i>	6.25	6.25	6.25	6.25	6.25	6.25	6.25	6.25	6.25	6.25	6.25	3.12	3.12	3.12	6.25	3.12	3.12	1.56	1.56	6.25	6.25	3.12	3.12	3.12	3.12
<i>Lippia javanica</i>	6.25	3.12	6.25	3.12	6.25	6.25	3.12	6.25	3.12	6.25	6.25	3.12	3.12	3.12	3.12	6.25	3.12	3.12	3.12	3.12	6.25	3.12	3.12	3.12	3.12
<i>Melianthus comosus</i>	3.12	3.12	3.12	6.25	1.56	0.78	0.78	3.12	3.12	1.56	0.78	0.78	1.56	6.25	0.78	0.78	0.19	1.56	6.25	0.78	0.39	0.19	1.56	3.12	0.39
<i>Pelargonium sidoides</i>	6.25	6.25	6.25	6.25	6.25	6.25	6.25	6.25	6.25	6.25	6.25	6.25	6.25	3.12	6.25	3.12	6.25	6.25	6.25	6.25	0.78	1.56	6.25	3.12	1.56
<i>Plectranthus ecklonii</i>	6.25	6.25	6.25	6.25	6.25	6.25	6.25	6.25	6.25	6.25	6.25	6.25	6.25	6.25	6.25	6.25	3.12	6.25	6.25	6.25	6.25	1.56	6.25	3.12	1.56
<i>Tetradenia riparia</i>	6.25	6.25	6.25	6.25	6.25	6.25	6.25	6.25	6.25	6.25	6.25	3.12	3.12	3.12	3.12	6.25	6.25	3.12	6.25	6.25	6.25	3.12	1.56	3.12	6.25
<i>Trichilia emetica</i>	6.25	6.25	6.25	6.25	6.25	6.25	6.25	6.25	3.12	3.12	6.25	3.12	3.12	3.12	3.12	6.25	6.25	6.25	3.12	3.12	6.25	3.12	3.12	3.12	3.12
<i>Vachellia karroo</i>	6.25	6.25	6.25	3.12	6.25	1.56	6.25	3.12	3.12	3.12	3.12	3.12	3.12	3.12	3.12	1.56	3.12	3.12	3.12	6.25	0.78	0.78	1.56	3.12	3.12
1% DMSO			6.25					6.25					6.25					6.25					6.25		
Ciprofloxacin			0.001					0.001					0.001					0.001					0.001		

1: Aqueous; 2: Methanol; 3: Acetone; 4: Ethyl acetate and 5: Dichloromethane.

dichloromethane (DCM) *M. comosus* extract showed a MIC of 1.56 mg/ml against *E. coli*. Aqueous and methanolic *M. comosus* extracts

both exhibited MICs of 0.78 mg/ml, whereas the *M. comosus* (DCM) extract indicated a MIC of 1.56 mg/ml against *K. pneumoniae*. *M. comosus*

(dichloromethane, aqueous and methanol) extracts with MIC value of 0.78 mg/ml showed significant activity, whereas acetone extract

displayed a MIC value of 1.56 mg/ml against *P. aeruginosa*. Ciprofloxacin (positive control) exhibited a noteworthy MIC value of 0.001 mg/ml against all five tested bacteria. The negative control (1% DMSO) against all strains validated that the solvent did not affect the activity. Mostly, the crude extracts showed insignificant activities against GNB and GPB (Table 3).

Qualitative and quantitative effect of plant extracts on violacein production

The fifty crude extracts tested for their QS inhibition (AQS potential) qualitatively using disc diffusion at various concentrations showed that only *M. comosus* (aqueous) exhibited activity by showing opaque zones of inhibition of 13, 14 and 15 mm at concentrations of 2 × MIC, 4 × MIC and 8 × MIC (1.56 - 6.25 mg/ml), respectively. This signaled the inhibition of violacein production controlled by the QS system in *C. violaceum*. The methanolic extracts of *M. comosus* exhibited bactericidal activity, showing clear zones of inhibition of 13 and 15 mm at concentrations of 4 × MIC and 8 × MIC, respectively. The other crude extracts were not capable of reducing the violacein production of *C. violaceum*. The positive control cinnamaldehyde also demonstrated only bactericidal activity with 13, 19, 13 and 26 mm clear inhibition zones at concentrations of 0.5, 0.25, 0.12 and 0.06 mg/ml, respectively. Based on this qualitative AQS screening, *M. comosus* (aqueous and methanol) extracts were the only extracts able to inhibit the violacein of *C. violaceum*.

According to our findings of qualitative AQS inhibition, some of the crude extracts exhibited no activity. Thus, the crude extracts were tested further for quantitative AQS assay and to confirm their violacein inhibition by verifying the precise concentration needed for bactericidal or QS inhibition. The sub-MIC concentration (0.097 - 1.56 mg/ml; $\frac{1}{8}$ MIC - 2 × MIC) reliant on the reduction of violacein production by *C. violaceum* was shown with 8 out of 50 crude extracts with inhibitory effects between 2.60 and 38.34%, as presented in Figure 1A. The indicated moderate activity (IC_{50} values) of crude extracts comparable to the positive control is shown in Table 4.

The aqueous extract of *M. comosus* and dichloromethane extract of *P. ecklonii* exhibited violacein inhibition at 2× MIC with 38.4% (IC_{50} = 1.52 mg/ml) and 25.91% (IC_{50} = 1.81 mg/ml), respectively. *A. arborescens* (acetone and ethyl acetate), *A. afra* (dichloromethane), *M. comosus* (acetone), *P. sidoides* (aqueous) and *P. ecklonii* (methanol) presented weak AQS activity with a reduced violacein inhibition between 2 and 20%. At the MIC concentration, *M. comosus* (aqueous) and *P. ecklonii* (dichloromethane) extracts exhibited violacein inhibition of 24.33 and 24.60%, respectively while most of the crude extracts had less than 10% inhibition. A decrease in concentration resulted in a reduced

violacein inhibitory effect at $\frac{1}{2}$ MIC, only methanolic and dichloromethane extracts of *P. ecklonii* showed 4.25 and 10.64%, respectively (Figure 1A). The other plant extracts have enhanced the growth of the bacterium (Figure 1B). The positive control, cinnamaldehyde also exhibited violacein reduction at 62.77% (MIC), 61.66% ($\frac{1}{2}$ MIC), 60.69% ($\frac{1}{4}$ MIC) and 59.43% ($\frac{1}{8}$ MIC), as shown in Figure 1A. The positive control (cinnamaldehyde) slightly reduced AQS activity, as it exhibited above 40% violacein inhibition at 0.048 mg/ml with an IC_{50} value of 0.087 mg/ml.

Biofilm formation inhibition of plant extracts against selected MDR pathogens

The two stages of cell attachment and biofilm development inhibition were evaluated for the six active extracts that showed significant MIC values against the five tested bacteria. These included *M. comosus* (dichloromethane, aqueous and methanol), *P. sidoides* (aqueous) and *V. karroo* (aqueous and methanol). The findings showed 4.33 to 66.34% antiadhesion inhibitory effects across the test pathogens (Table 5). Good antiadhesion inhibition was observed with the following extracts of *M. comosus* (methanol) and *P. sidoides* (aqueous), with percentage inhibition of 50.28 and 66.34%, respectively. The other four extracts of *M. comosus* (aqueous and dichloromethane) and *V. karroo* (aqueous and methanol) showed weak biofilm inhibition between 0.64 and 49.53% against *S. pyogenes*. The positive control, ciprofloxacin, exhibited very good inhibitory activity of 72.53% against *S. pyogenes*. The methanolic extract of *M. comosus* showed inhibitory activity of 40.27% while the other five crude extracts exhibited ± 30% weak inhibitory effects against *S. aureus*, while ciprofloxacin showed 61.34% inhibitory activity.

The tested GNB (*P. aeruginosa*, *E. coli* and *K. pneumoniae*) presented some degree of resistance towards the crude extracts. Four extracts displayed weak inhibition of 0.64 to 29.12% against *K. pneumoniae*, while two extracts enhanced the growth or adhesion of the bacterium (Table 5). Furthermore, all crude extracts enhanced the growth of *E. coli* and *P. aeruginosa* instead of anti-adhesion inhibition, while ciprofloxacin exhibited 53.49 and 52.88% inhibitory activity against *E. coli* and *P. aeruginosa*, respectively.

The six crude extracts were also assessed for their potential to reduce the mature biofilms of the five tested MDR bacteria. The crude extracts presented weak activity between 8.54 and 31.82% against the five tested bacteria (Table 5). The effects of plant extracts on biofilms were observed only on GNB whereas GNB were resistant to treatment but rather enhanced growth. Aqueous *P. sidoides* and *M. comosus* (aqueous, methanol and dichloromethane) extracts showed

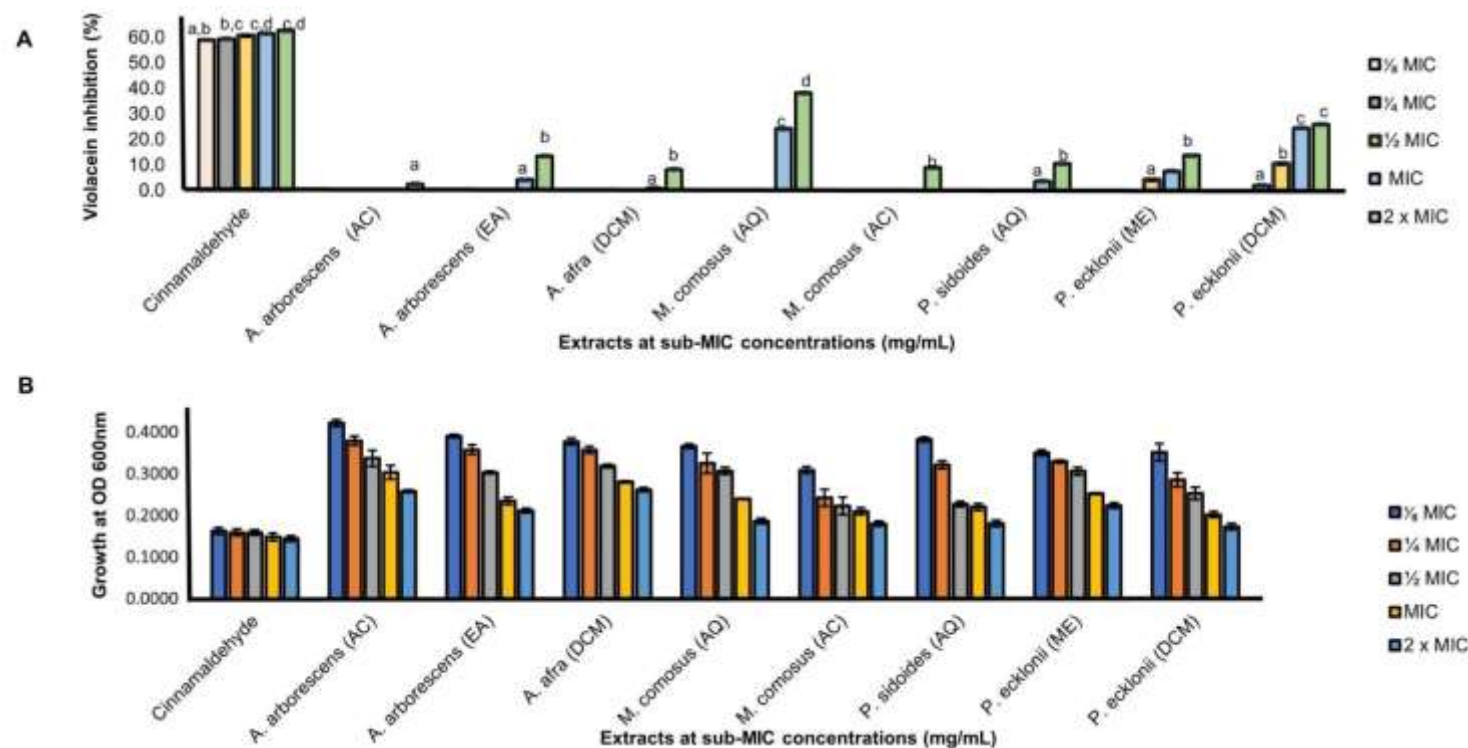


Figure 1. (A) Quantitative violacein inhibition (%) using crude extract against *C. violaceum* at concentrations between 0.190 -1.56 mg/ml ($2 \times \text{MIC}$ - $\frac{1}{8} \text{MIC}$). The results are shown as the percentage of violacein inhibition. A 100% production was set as control (untreated). Statistical significance of each test across concentrations with different letters (a–d) reflects statistically significant with $p < 0.05$. **(B)** Growth kinetics of *C. violaceum* treated with various plant extracts at sub-MIC concentrations (indicated on the graph) after 24 hrs. Means are values of triplicate independent experiments $\pm$ SD. AQ: aqueous, ME: methanol, AC: acetone, EA: ethyl acetate and DCM: dichloromethane.

percentage inhibition of 8.54, 9.81, 31.82 and 31.16%, respectively against *S. pyogenes*. Methanolic and dichloromethane extracts of *M. comosus* were the only extracts that exhibited weak activities of 10.97 and 17.45% against *S. aureus*. While none of the crude extracts exhibited any inhibitory effect for *E. coli*, *K. pneumoniae* or *P. aeruginosa*. Ciprofloxacin showed some degree of inhibition against *E. coli*, *K. pneumoniae* and *P. aeruginosa*, with 36.27,

39.72 and 39.43%, respectively.

The results are presented as the percentage inhibition of cell attachment and biofilm development.

GC-MS profiling of crude extracts

The gas chromatograms and the identified peaks of the different plant extracts of *P. sidoides*

(aqueous) and *V. karroo* (aqueous and methanol) are shown in Figure 2 and Table 6, respectively. Baloyi et al. (2021) reported the chemical profiling of *M. comosus* extracts. According to the library search for the identification of chemical constituents, the aqueous extract of *P. sidoides* showed eight compounds: butanoic acid (2), propanoic acid (3), 2-ketobutyric acid (5), linalool octadecanoic acid (14) and octadecanoic acid, 2,3-propyl ester (14). The aqueous extract of *V.*

Table 4. Inhibitory concentration (IC₅₀) values of potent plant extract against *C. violaceum*.

Plant species	Half Y	IC ₅₀ (mg/ml)
Aqueous extract		
<i>Melianthus comosus</i>	37.66	1.52
<i>Pelargonium sidoides</i>	9.70	1.45
Methanol extracts		
<i>Plectranthus ecklonii</i>	9.80	1.05
Acetone extracts		
<i>Aloe arborescens</i>	11.51	2.49
<i>Melianthus comosus</i>	11.41	2.48
Ethyl acetate extract		
<i>Aloe arborescens</i>	12.07	1.43
Dichloromethane extracts		
<i>Artemesia afra</i>	10.23	2.24
<i>Plectranthus ecklonii</i>	27.65	1.81
Cinnamaldehyde	56.35	0.087

karroo comprises seven constituents: 1,2,3-propanetriol (1), 2-ketobutyric acid (5), 2-aminobutanoic acid (6), 1,2,3-benzenetriol (7), decanoic acid (8), hexadecanoic acid (13), and octadecanoic acid (14) whereas the methanolic extract of *V. karroo* comprises five compounds, 1,2,3-propanetriol (1), 1-butanol, 3-methyl-, acetate (4), α -D- glucopyranoside, methyl (10), hexadecanoic acid (13), and octadecanoic acid (14) with α -D-glucopyranoside, methyl as the major constituents.

DISCUSSION

For more than 5000 years communities have been using natural products for treating infections, as the indigenous plants provide bioactive phytochemical compounds that lead to the development of novel therapeutics (Anand et al., 2019). Approximately ten medicinal plants were collected based on their biological activities and reported medicinal uses, as presented in Table 1. These plants were chosen based on their known medicinal usage for the treatment of septicaemia, urinary tract infection, respiratory diseases, wound and skin diseases infections including their reported antibacterial, anti-inflammatory, antifungal and antidiabetic activities.

The extraction process is vital for sourcing various bioactive phytochemicals from medicinal plants; hence, we used solvents of different polarities. This is further substantiated by the fact that the solubility of plant extracts differs per solvent and relative to the plant part used for a variety of phytoconstituents. Here, we have shown that aqueous and methanol extracts have higher

yields than ethyl acetate, dichloromethane and acetone. Similar to our study, Eloff et al. (2018) and Mabona et al. (2013) indicated that methanol and water had higher percentage extraction yields. Thus, larger yields do not mean better biological activity, as there are different proportions of compounds present in a plant.

Traditional healers and the community of South Africa at large often use medicinal plants as an alternative herbal medicine for infectious diseases. This has led to researchers studying plants for their antimicrobial properties, specifically with the reference strains, which more or less corresponds with literature (Elisha et al., 2017; van Vuuren and Muhlarhi, 2017; Shirinda et al., 2019). The efficiency of plant extracts as potential antibacterials may be attributed to plants continuously protecting themselves against phytopathogens that are within their environment. This triggers the production of broad-spectrum compounds with antimicrobial activity (Nguta and Kiraithe, 2019).

To validate the antibacterial activities of the ten medicinal plants, a noteworthy activity of a plant extract was regarded when the MIC value was 1 mg /ml or lower (van Vuuren and Muhlarhi, 2017). Susceptibility against *S. aureus*, *P. aeruginosa*, *E. coli*, *S. pyogenes* and *K. pneumoniae* was observed for *Artemesia afra* (acetone and ethyl acetate), *M. comosus* (aqueous, methanol, acetone and dichloromethane), *T. riparia* (acetone), *P. ecklonii* (methanol and dichloromethane), *P. sidoides* (aqueous, methanol and dichloromethane) and *V. Karroo* (aqueous, methanol and acetone) extracts showing noteworthy MIC values (Table 3).

Compared to our study, van Vuuren and Muhlarhi

Table 5. Percentage inhibitory effects of plant extracts on cell attachment and biofilm development against MDR pathogens

Plant species	Cell attachment inhibition (%)				
	<i>E. coli</i> ATCC 10536	<i>K. pneumonia</i> ATCC 33495	<i>P. aeruginosa</i> ATCC 9721	<i>S. aureus</i> ATCC 25923	<i>S. pyogenes</i> ATCC 19615
Aqueous extracts					
<i>M. comosus</i>	-	23.42±0.05 ^{a,b}	-	31.70±0.06 ^{a,b}	46.61±0.10 ^{b,c}
<i>P. sidoides</i>	-	9.17±0.05 ^a	-	39.60±0.06 ^{a,b}	66.34±0.22 ^c
<i>V. karroo</i>	-	-	-	32.09±0.06 ^{a,b}	40.38±0.10 ^b
Methanol extracts					
<i>M. comosus</i>	-	29.12±0.05 ^{a,b}	-	40.27±0.08 ^b	50.28±0.12 ^{b,c}
<i>V. karroo</i>	-	-	-	4.33±0.05 ^a	38.05±0.06 ^b
Dichloromethane extract					
<i>M. comosus</i>	-	0.64±0.05 ^a	-	35.34±0.06 ^b	49.53±0.10 ^{b,c}
Ciprofloxacin	53.48±0.18 ^{b,c}	59.30±0.15 ^{b,c}	52.88±0.18 ^{b,c}	61.34±0.20 ^c	72.53±0.22 ^c
Biofilm development inhibition (%)					
Aqueous extracts					
<i>M. comosus</i>	-	-	-	-	9.81±0.05 ^a
<i>P. sidoides</i>	-	-	-	-	8.54±0.05 ^a
<i>V. karroo</i>	-	-	-	-	-
Methanol extracts					
<i>M. comosus</i>	-	-	-	10.97±0.05 ^a	31.82±0.06 ^{a,b}
<i>V. karroo</i>	-	-	-	-	-
Dichloromethane extract					
<i>M. comosus</i>	-	-	-	17.45±0.05 ^a	31.16±0.06 ^{a,b}
Ciprofloxacin	39.72±0.06 ^b	36.27±0.06 ^b	39.43±0.06 ^b	40.90±0.08 ^b	49.02±0.10 ^{b,c}

The results are presented as the percentage inhibition of cell attachment and biofilm development. (-) No inhibition. Means are values of triplicate independent experiments ± SD. The contrast of percentage inhibition at 1 mg/ml for every sample against GPB (*S. pyogenes* and *S. aureus*) and GNB (*P. aeruginosa*, *E. coli* and *K. pneumoniae*). Various letters (a–c) show significant differences at $p < 0.05$. *M. comosus*: *Melianthus comosus*; *P. sidoides*: *Pelargonium sidoides* and *V. karroo*: *Vachellia karroo*. AQ: aqueous, ME: methanol, DCM: dichloromethane.

(2017) reported higher MIC values (>8.00 mg/ml) for aqueous extracts of *A. afra*, *L. javanica* and *T. riparia* against *E. coli*, *K. pneumoniae*, *P.*

aeruginosa and *S. aureus*. Our study also reported improved MIC values of 6.25 mg/ml for aqueous extracts of *A. afra*, *L. javanica* and *T.*

riparia against the four tested pathogens. Mulaudzi et al. (2011) indicated a significant MIC value of 0.195 mg/ml for *V. karroo* (aqueous)

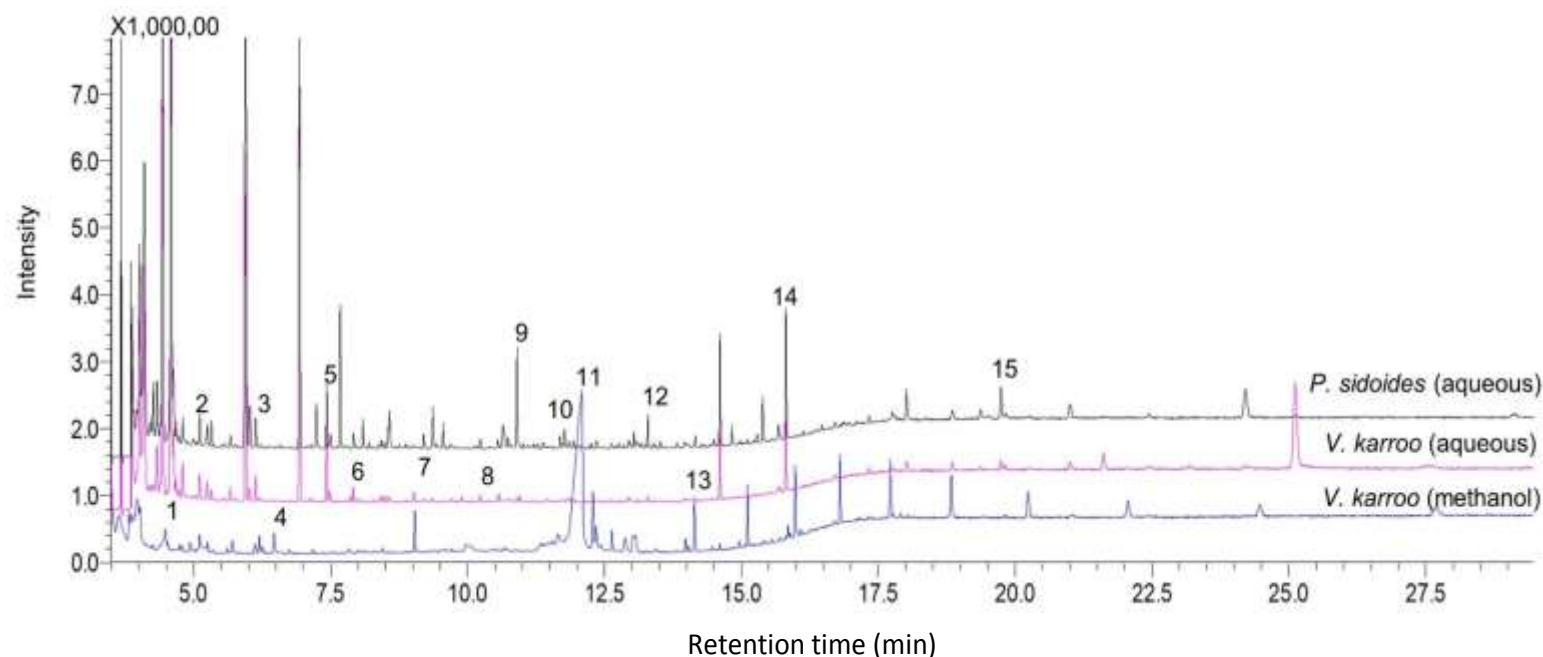


Figure 2. GC-MS total ion chromatograph of *P. sidoides* (aqueous) and *V. karroo* (aqueous and methanol) extracts. Peak numbers and their identities correspond to those in Table 6.

extract against *S. aureus*, while our study showed an aqueous extract of *V. karroo* had MIC value of 1.56 mg/ml against *S. aureus*.

Incongruent with our study, Aboobaker et al. (2019) reported weak activities between 1.00 and 4.00 mg/ml of aqueous and organic extracts of *P. sidoides* whereas our study reported a similar MIC value of 6.25 mg/ml for *P. sidoides* (aqueous and organic) extracts against *S. aureus*. Based on our results, an aqueous extract of *P. sidoides* exhibited good activity (0.78 mg/ml) against *S. pyogenes*.

Contrary to our findings, Kelmanson et al. (2000) reported no bacterial activity of the *M. comosus* (methanol) extract against *P.*

aeruginosa and *E. coli*, while only *S. aureus* showed a MIC value of 2.00 mg/ml, whereas Mabona et al. (2013) reported MIC values of 1.60 and 2.00 mg/ml against *S. aureus* and *P. aeruginosa*, respectively. The most effective plant species indicating significant activity against the resistant strains was *M. comosus*, followed by *V. karroo* > *P. sidoides* > *P. ecklonii* and *A. afra*. According to van Vuuren and Muhlarhi (2017), Mamabolo et al. (2018) crude plant extracts showing MIC values >1 mg/ml, are regarded as noteworthy. On that basis, our extracts exhibited good activities and could be promising natural antibacterials for the studied test pathogens.

Generally, Gram-positive bacteria (*S. aureus* and *S. pyogenes*) are more susceptible to the treatment of plant extracts than Gram-negative bacteria (*E. coli*, *K. pneumoniae* and *P. aeruginosa*) which demonstrate moderate activities. This is attributed to the inability of plant extracts to penetrate through the outer membrane (OM) of Gram-negative bacteria due to the structural lipopolysaccharide component (De Zoysa et al., 2019). No antibacterial activity does not signify inactive bioactive compounds within the plants; instead, inadequate quantities of phytochemicals in the extract could be the reason for poor activity (De Zoysa et al., 2019). The results obtained in this study confirmed the effective use of these

Table 6. Phytochemical constituents identified from *P. sidoides* (aqueous) and *V. karroo* (aqueous and methanol) extracts using GC-MS.

Peak	Ret. time (min)	Name	Molecular weight	Molecular formula	Plant extracts		
					<i>P. sidoides</i> (AQ)	<i>V. karroo</i> (AQ)	<i>V. karroo</i> (ME)
1	4.63	1,2,3-Propanetriol	C ₃ H ₈ O ₃	92	-	0.8	0.5
2	5.324	Butanoic acid	C ₇ H ₁₆ O ₂ Si	160	0.4	-	-
3	6.027	Propanoic acid	C ₉ H ₂₂ O ₃ Si ₂	234	0.8	-	-
4	6.435	1-Butanol, 3-methyl-, acetate	C ₇ H ₁₄ O ₂	130	-	-	1.2
5	7.428	2-Ketobutyric acid	C ₉ H ₁₉ NO ₃ Si	217	1.0	1.4	-
6	7.916	2-Aminobutanoic acid	C ₁₀ H ₂₅ NO ₂ Si ₂	247	-	0.2	-
7	9.515	1,2,3-Benzenetriol	C ₆ H ₆ O ₃	126	-	0.3	-
8	10.235	Decanoic acid	C ₁₃ H ₂₈ O ₂ Si	244	-	0.1	-
9	10.435	Linalool	C ₁₀ H ₁₈ O	154	3.2	-	-
10	10.903	Pyroglutamic acid	C ₅ H ₇ NO ₃	129	1.7	-	-
11	11.835	α-D-Glucopyranoside, methyl	C ₇ H ₁₄ O ₆	194	-	-	44
12	13.294	Tetradecanoic acid	C ₁₇ H ₃₆ O ₂ Si	300	0.5	-	-
13	14.575	Hexadecanoic acid	C ₁₉ H ₄₀ O ₂ Si	328	-	1.0	0.6
14	15.815	Octadecanoic acid	C ₂₁ H ₄₄ O ₂ Si	356	1.9	1.1	0.5
15	19.743	Octadecanoic acid, 2,3-propyl ester	C ₂₇ H ₅₈ O ₄ Si ₂	502	0.9	-	-

extracts; thus, this instigated further investigation on the antipathogenic properties of the extracts.

As mentioned earlier, plant extracts continuously demonstrate bactericidal activity; however, few reports have explored their ability to interfere with the QS signaling mechanism. Targeting QS signaling emerges as an innovative strategy, as this mechanism focuses on virulence factors such as biofilms and others, tackling pathogenic bacteria and thereby disarming them (Yüzbaşıoğlu et al., 2017).

A previous study by Alreshidi et al. (2020) showed that using the disc diffusion assay, the *T. polium* methanolic extract had no inhibitory effects against *C. violaceum*. Based on the above results (Figure 1A), plant extracts have some inhibitory effect at a higher concentration (2 ×

MIC) while at a lower concentration (1/8 MIC); there were insignificant inhibitory effects of plant extracts on violacein production. Comparable to our study, Yüzbaşıoğlu et al. (2017) documented plant extracts of Turkey origin to slightly reduce the violacein production of *C. violaceum* ATCC 12472 at a sub-MIC concentration (0.25 mg/ml). Bodede et al. (2018) reported a 14.2 to 28% QSI of six crude extracts of *S. nigrescens* at a concentration of 0.6 mg/ml. These findings validate that plant extracts possess AQS/QSI activities at sublethal concentrations. Vatter et al. (2007) mentioned that natural products or extracts inhibit the accumulation of QS signals by reducing the concentration of active signal molecules either enzymatically or nonenzymatically. Furthermore, this can also be accomplished by

phytochemicals competitively or noncompetitively binding to the receptor protein.

Thus far, research studies have made it completely clear that natural products, as well as medicinal plants, are a promising source of AQS/QSI compounds. The present study revealed that the leaf parts of *M. comosus*, *P. ecklonii* and *P. sidoides* exert bacteriostatic and/or antipathogenic activities against *C. violaceum* which may promote the possibility of the plant's defense mechanism against diverse bacterial invasions. Notably, the plant extracts of *V. karroo* exhibited significant antibacterial activities against Gram-negative bacteria and Gram-positive bacteria but did not show any AQS activities; thus, these plant extracts were capable of exhibiting good potency against

MDR bacteria through different mechanisms of action.

The findings of this study indicated good activity of the plant extracts to inhibit the initial stage of biofilm formation, especially on the Gram-positive bacteria of *S. aureus* and *S. pyogenes*. This could be due to the ability of extracts to restrict forces such as Van der Waals forces and electrostatic interactions that promote attachment to the surfaces of the bacteria (Famuyide et al., 2019). As mentioned earlier, the Gram-negative bacteria (*E. coli*, *K. pneumoniae* and *P. aeruginosa*) showed weak to no inhibitory activity of the plant extracts because the three-component membrane was resistive to the diffusion of the extracts (Liu et al., 2019).

Several reports have corroborated that the complete removal of biofilms is challenging, as numerous biofilm-forming microbes showed resistance towards treatment (Baloyi et al., 2019; Cosa et al., 2020; Sarkar et al., 2015). This could be because when biofilms are in their natural surroundings, they include multiple species, whereby the interspecies may be the contributing factor to the growth and formation of this consortium of microorganisms (Giaouris et al., 2015). The mechanism of MDR bacteria is multifactorial, thus this is not fully understood and requires further investigation (Guzzo et al., 2020). Furthermore, Giaouris et al. (2015) indicated that QS communication between bacteria in a biofilm has an impact on their relative resistance.

Medicinal plants of *M. comosus*, *P. sidoides* and *V. karroo* warrant further investigation for the identification of the active compounds. From the antibiofilm activity for the antiadhesion and biofilm development stages (Table 5), these extracts revealed diverse results. The ability of extracts to reduce biofilm activity could be the result of the quorum sensing process of pathogenic bacteria by either impeding with autoinducer synthesis or autoinducer reception and transduction or degrading autoinducers (Theodora et al., 2019). Because *M. comosus*, *P. sidoides* and *V. karroo* extracts were most potent in inhibiting bacterial growth of Gram-negative bacteria and Gram-positive bacteria and potentially inhibited QS-dependent violacein production, the plants were then chosen for further analysis of the chemical compounds using GC-MS.

In this study, the GC-MS method was chosen to distinguish the phytoconstituents found in *P. sidoides* and *V. karroo* and revealed that saturated fatty acids of octadecanoic acid and hexadecanoic acids are commonly known as stearic and palmitic acid, respectively. These bioactive compounds are described to have antibacterial activity against *P. aeruginosa* and *S. aureus* (Ivanova et al., 2017). Chemical characterization of *P. sidoides* has shown that the plant has a rich source of tannins, fatty acids, phenolics, coumarins and flavonoids (Kolodziej, 2007).

Several studies (Maroyi, 2017a; Nyila et al., 2012; Priyanka et al., 2015) reported phytochemical analysis of various plant parts of *V. karroo*, revealing various classes

of compounds, such as terpenes, tannins, phenols, proanthocyanidin and flavonoids. Priyanka et al. (2015) identified various constituents of compounds from the roots and leaves of *V. karroo* (chloroform, ethanol, and ethyl acetate) using GC-MS analysis. Several efficient methods for the identification of bioactive compounds is GC-MS, which can detect diverse classes of compounds, such as steroids, alkaloids, organic acids, esters, hydrocarbons, amino acids and alcohols (Konappa et al., 2020).

Conclusion

The findings of the study validated the antibacterial activity of *A. afra*, *P. ecklonii*, *P. sidoides*, *V. karroo* and *M. comosus* extracts. This further highlighted the significance of assessing the unexplored AQS properties of medicinal plants. *M. comosus*, *P. sidoides* and *P. ecklonii* have shown the potential to disrupt violacein production of *C. violaceum*. Notably, *M. comosus*, *P. sidoides* and *V. karroo* have also demonstrated good anti-adhesion activity against Gram-positive bacteria of *Staphylococcus aureus* and *Streptococcus pyogenes* and impeded a slight inhibition of Gram-negative bacteria formed biofilms. This study was able to predict the structures and chemical formulas of secondary metabolite components present in plant extracts of *P. sidoides* and *V. karroo*. Thus, plant bioactive compounds could contribute to restricting the development of bacteria within infected hosts through the disruption of bacterial virulence, weakening the potency of pathogenic bacteria. For future purposes, this renders significant growth in discovering a treatment for infectious diseases.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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