An in vitro study on the potential of selected South African plant extracts to prevent and treat bovine mastitis

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Abstract

Mastitis is a painful inflammatory condition, commonly resulting from infections caused by *Staphylococcus aureus* and other pathogens. In this study, antimicrobial, anti-biofilm and anti-quorum sensing activities of selected plants were investigated against *Staphylococcus aureus* isolates from clinical cases of bovine mastitis. Acetone and ethanol extracts of 15 plants were initially screened for their antimicrobial activities against the commercially available reference strain *S. aureus* ATCC 29213. *Maytenus undata*, *Maurocenia frangula*, *Kalanchoe gunniae* and *Bryophyllum pinnatum* had good activities and extracts were tested against six multi-drug resistant *S. aureus* strains isolated from clinical bovine mastitis cases. The four plants were further analysed for cytotoxicity and anti-quorum sensing activities. Two of the plants with good results were assessed further for anti-biofilm activity. Minimum inhibitory concentration (MIC) values of the extracts against *S. aureus* isolates ranged from 0.02 to 0.63 mg/mL. *Kalanchoe gunniae* extracts were the least cytotoxic with extremely promising selectivity index (SI) values ranging from 12 to 25. Significant inhibition of biofilm development against *S. aureus* mastitis isolates was observed, but extracts were not able to eradicate pre-formed biofilms. *Kalanchoe gunniae* extracts had the best anti-quorum sensing activity with minimum quorum sensing inhibition concentration (MQSIC) and MIC values of 0.04 mg/mL and 0.63 mg/mL respectively against *Chromobacterium violaceum* ATCC 12472. Therefore, *K. gunniae* holds potential for development of a preventative treatment against bovine mastitis.

Keywords: Celastraceae; Crassulaceae; *Kalanchoe gunniae;* mastitis; *Staphylococcus aureus;* antimicrobial; cytotoxicity; anti-biofilm; anti-quorum sensing

1. Introduction

Infectious diseases caused by microorganisms are a global public health problem (Cos et al., 2006; Jiménez-González et al., 2013). One such infectious disease is mastitis, which occurs in humans and dairy cows. In cattle the disease is characterized by swelling, tenderness, redness of the skin, as well as warm and painful udders (Semba, 2000; Sharma et al., 2011; Sserunkuma et al., 2017). It leads to a decrease in milk production as well as low quality of milk, resulting in huge financial losses (Banga et al., 2014). Bacteria, followed by fungi and in very rare cases

algae and viruses have been reported as causative organisms (Bourtzi-Hatzopoulou et al., 2003; Viguier et al., 2009). Among the bacterial agents, *Staphylococcus aureus* is predominant (Harmon, 1994; Waage et al., 1999), mainly due to its intracellular nature and a high prevalence of resistance to antimicrobials (Goni et al., 2004).

Initially, penicillin and methicillin were used effectively for the treatment of *S. aureus* infections; however, resistance to these antimicrobials has emerged (Parker and Jevons, 1964; Smith et al., 1999; Saga and Yamaguchi, 2009). Of great concern is the presence of mastitis caused by methicillin-resistant *S. aureus,* or MRSA, which is not responsive to readily available intramammary antimicrobials (Ateba et al., 2010; Pexara et al., 2013). Staphylococci, including particularly *S. epidermidis* and *S. aureus*, are among the best studied clinically relevant biofilm-forming organisms, aiding in their ability to resist antimicrobial treatment (Otto, 2008). Together with the inherent and acquired mechanism of resistance, bacteria have the ability to adhere to both biotic and abiotic surfaces to form protective biofilms (Sandasi et al., 2008). Another mechanism of antimicrobial resistance relates to quorum sensing (QS) which regulates expression of virulence factors, adhesion, biofilm formation, exopolysaccharide production, and sporulation in different microorganisms (Chenia, 2013). The QS mechanism has also been linked to the ability of the organism to develop multiple drug resistance (Padder et al., 2018). This further limits prescription choice and impacts on patient prognosis (Rasko and Sperandio, 2010).

Plant extracts have been reported to inhibit bacterial cell attachment, therefore limiting the ability of the organism to form biofilms. This is likely to reduce microbial colonization and control infection (Sandasi et al., 2010). For this study, fifteen plant species were selected based on chemotaxonomy and ethnopharmacology. In the study of Pauw and Eloff (2014), it was reported that species belonging to the order Celastrales had promising antimicrobial activity against bacteria and fungi. Twelve species from the Celastraceae family were thus chosen for this study. A further three plant species were selected based on ethnopharmacological use. From the Aquifoliaceae family, *Ilex mitis* (stem bark) is used traditionally in South Africa to treat skin infections/rashes, rheumatism, sores and fever (Mabona et al., 2013; Sagbo and Mbeng, 2018). From the Crassulaceae family, *Bryophyllum pinnatum* (also known as *Kalanchoe pinnata*) (Okwu and Uchenna, 2009; Okwu and Nnamdi, 2011; Chibli et al., 2014)

and *Kalanchoe gunniae* were selected. *Bryophyllum pinnatum* is used ethnopharmacologically in Nigeria, southern India, and Bengal to treat abscesses, skin ulcer, burns, earache, gonorrhoea, skin infections, inflammation, fever and cough (Okwu and Nnamdi, 2011; Nagaratna and Hegde, 2015). In Southeast Nigeria in particular, juice from the leaves of *B. pinnatum* is used traditionally to facilitate the dropping of remains of umbilical cord from newborn babies as well as for prevention of naval infection and facilitation of naval healing of babies (Okwu and Uchenna, 2009). *Kalanchoe gunniae,* a hybrid plant growing in South Africa, was only described in 2019 (Smith et al., 2019) Since it is related to *Bryophyllum pinnatum* (*K. pinnata*), it is hypothesised that *Kalanchoe gunniae* could possibly also have antimicrobial activity, and therefore it was included in this research.

The aim of this study was to investigate the antibacterial efficacy of selected plant species against a reference ATCC strain of *S. aureus* (ATCC 29213) and *S. aureus* isolates from clinical mastitis cases. Furthermore, their cellular safety was investigated against two mammalian cell lines. Their ability to inhibit bacterial quorum sensing was also investigated. Plant extracts that were active in all the above-mentioned bioassays as well as non-cytotoxic were further investigated for their effect on biofilm formation.

2. Materials and methods

2.1. Plant collection and processing

Leaves of *Catha edulis* and leaves and stem bark of *Ilex mitis* and *Kalanchoe gunniae* were collected from the Pretoria Botanical Garden, South African National Biodiversity Institute (SANBI), South Africa. Leaves of *Maytenus undata, Mystroxylon aethiopicum* and *Gymnosporia buxifolia* were collected from the Walter Sisulu Botanical Garden, SANBI, South Africa. Leaves and stem bark of *Bryophyllum pinnatum* were collected from the Federal Institute of Industrial Research Oshodi, Lagos Nigeria, Medicinal Plant Garden. Leaves of *Gymnosporia heterophylla, Elaeodendron transvaalense, Gymnosporia senegalensis, Elaeodendron croceum, Maurocenia frangula, Maytenus peduncularis, Robsonodendron eucleiforme* and *Pseudosalacia streyi* were collected from the Lowveld National Botanical Garden, SANBI, Nelspruit, Mpumalanga, South Africa.

Herbarium specimens were prepared and deposited at the H.G.W.J. Schweickerdt Herbarium, University of Pretoria (PRU) and the National Herbarium (SANBI) Pretoria (PRE), South Africa (Table 1). Using the method described by Eloff and McGaw (2006), harvested plant materials were cleaned, and hung inside open mesh loose woven bags and dried indoors at room temperature. The succulent plant material of *Bryophyllum pinnatum* and *K. gunniae* was dried by cutting into pieces and placing inside a constant air flow dehumidifying oven dryer at 33^{o}C. When dried, plant material was ground to fine powder using a Merck MF 10 Basic plant mill, and stored in closed glass jars at room temperature in the dark as recommended by Eloff and McGaw (2006). The ground plant materials were stored overnight and extracted the following day.

Table 1. Plants investigated in this study

Plant species by family	Part collected	Voucher number
Celastraceae		
Maytenus undata (Thunb.) Blakelock	Leaves	PRU 125486
Mystroxylon aethiopicum (Thunb.) Loes.	Leaves	PRU 125488
Catha edulis (Vahl) Endl.	Leaves	PRU 125485
Gymnosporia buxifolia (Eckl. and Zeyh.) Loes.	Leaves	PRU 125487
Gymnosporia heterophylla (Eckl. and Zeyh.) Loes.	Leaves	PRE1004259
Elaeodendron transvaalense (Burtt Davy) R.H.Archer	Leaves	PRE 1004258
Gymnosporia senegalensis (Lam.) Loes.	Leaves	PRE 1004261
Elaeodendron croceum (Thunb.) DC.	Leaves	PRE 1004265
Maurocenia frangula Mill.	Leaves	PRE 1004262
Maytenus peduncularis (Sond.) Loes	Leaves	PRE 1004264
Robsonodendron eucleiforme (Eckl. and Zeyh.) R.H.Archer	Leaves	PRE 1004268
Pseudosalacia streyi Codd	Leaves	PRE 1004260
Aquifoliaceae		
Ilex mitis (L.) Radlk.	Leaves, stem bark	PRU 125483
Crassulaceae		
Kalanchoe gunniae Gideon F.Sm. and Figueiredo	Leaves, stem bark	PRE 1004266
Bryophyllum pinnatum (Lam.) Oken (syn. *Kalanchoe pinnata* (Lam.) Pers.)	Leaves, stem bark	PRE 1004263

2.2. Plant material extraction

Acetone and ethanol were used for extraction of plant material. Acetone purity was ≥99% and ethanol purity was 99.9%. Both solvents were purchased from Minema Chemicals (Pty) Ltd, South Africa. Acetone is able to extract a broad range of polar and non-polar compounds, and has been recommended as the best extractant to use in preparing plant extracts for antibacterial

testing (Eloff, 1998a). Ethanol is able to extract more polar compounds from plant material, and is often preferred for use in preparing samples for formulation and product development as it is less flammable than acetone (Gupta et al., 2012).

For extraction, separate aliquots (4 g of ground plant material) were weighed into 50 mL Falcon tubes. Using a plant material to solvent ratio of 1:10, 40 mL of either acetone or ethanol was added into each tube. The tubes containing the plant samples were shaken vigorously for 25 min with a mechanical shaker and sonicated for 15 min. Tubes containing the samples were shaken vigorously again for 2 min and vortexed for 1 min. Ultrasonication of samples were again done for another 5 min. The supernatants containing the extracts were collected into a glass beaker by filtering the extracts through Whatman No 1 filter paper. The process was repeated two times with the plant material residue (marc) using half the volume of starting solvent. For each sample, the supernatants containing the extracts were combined and concentrated to dryness by evaporating the solvent with continuous flow of air at 30^{o}C. When the samples were completely dried, the extract yield and percentage extract yield were calculated.

2.3. Bacterial strains and culturing

Staphylococcus aureus (ATCC 29213) and *Chromobacterium violaceum* (ATCC 12472) were obtained from the culture collection of the Phytomedicine Programme, Department of Paraclinical Sciences, Faculty of Veterinary Sciences, University of Pretoria. *Staphylococcus epidermidis* (ATCC 35984) and *S. aureus* mastitis clinical isolates; STA 1, STA 2, STA 3, STA 4, STA 5, and STA 6 were provided by the Milk Laboratory, Department of Production Animal Studies, Faculty of Veterinary Sciences, University of Pretoria. The bacteria were isolated from routine milk examinations and signed permission from all clients was obtained to use the samples for research. The ethical approval number for collection of bovine mastitis isolates provided by the University of Pretoria Ethics Committee is V121-16. *Staphylococcus* species were maintained on Mueller-Hinton (MH) agar plates and prior to the bioassays were grown in MH liquid media overnight at 37^{o}C with shaking. *C. violaceum* was maintained on Luria–Bertani (LB) agar at room temperature of 25^{o}C (±2^{o}C) and when needed for experiments, single colonies were cultured in LB broth overnight at 30^{o}C with shaking.

2.4. Antimicrobial susceptibility testing

Antibiogram assays to determine the susceptibility of the bacterial isolates were performed using the disc diffusion method (Bauer et al., 1966) using eight antibiotics belonging to six classes. These included: penicillins (ampicillin 10 µg and amoxicillin/clavulanic acid 30 µg), cephalosporins (ceftiofur 30 µg), tetracyclines (doxycycline 30 µg and tetracycline 30 µg), aminoglycosides (amikacin 30 µg), sulfonamides (sulphamethoxazole/trimethoprim 25 µg) and amphenicols (chloramphenicol 30 µg). Standardized bacterial suspensions of the micro-organisms were made in normal saline. This was done by picking a single colony from bacterial strains cultured overnight on MH agar at 37°C and suspending it in ±5 mL normal saline in tubes to obtain an inoculum equivalent to 0.5 McFarland standard. Each tube was gently vortexed to obtain a homogeneous suspension. Sterile cotton swabs were used to streak the suspensions of the microorganisms on agar plates (each comprising 25 mL MH agar). Antibiotic discs (Oxoid) were dispensed on inoculated agar plates which were incubated overnight at 37°C. Bacterial strains tested include *S. aureus* ATCC 35984 and the six *S. aureus* isolates from clinical mastitis (STA 1 to STA 6). After incubation, the zones of inhibition were measured according to the performance standard of the Clinical and Laboratory Standards Institute for antimicrobial disc susceptibility tests for bacteria isolated from animals (CLSI, 2020).

2.5. Antibacterial screening

Antibacterial activity of each of the plant extracts was first assayed against *S. aureus* (ATCC 29213). After this initial screening of all the plant extracts, the two most active plants from the chemotaxonomy selection and the two most active plants from ethnopharmacological selection were further assayed against six mastitis clinical isolates of *S. aureus*. The broth microdilution method (Eloff, 1998b) was used and the minimum inhibitory concentration (MIC) of the extracts was determined as previously described (Dzoyem et al., 2014). Stock solutions of extracts (10 mg/ml) were prepared in 50% acetone and 50% ethanol for acetone and ethanol extracts respectively. One hundred µl of 10 mg/ml samples were added to the first wells of each column of a 96-well microtitre plate, with a final concentration range from 2.5 to 0.0195 mg/mL. Gentamicin was used as a positive control and negative solvent controls were also included. MIC values were determined visually as a reduction in red colour of the formazan salt, indicating inhibition of bacterial growth. The assays were done in duplicate, each with

triplicate samples. The total antibacterial activity (TAA) in mL/g was determined by dividing the extract yield of 1 g of plant material in mg by MIC in mg/mL (Eloff, 2000).

2.6. Cytotoxicity

The 3-(4,5-dimethylthiazol)-2,5-diphenyl tetrazolium bromide (MTT) assay method of Mosmann (1983) was used to investigate the cytotoxicity of the eight most active plant extracts on Vero monkey kidney cells and bovine dermis cells with modifications as previously described (Olawuwo et al., 2022). Plant extracts used to treat the cells were first dissolved in dimethyl sulphoxide (DMSO) to a concentration of 100 mg/mL. From this concentration, different concentrations of the plant extracts ranging from 1 to 0.0075 mg/mL were prepared using MEM. Each of the different concentrations (100 µL) was added to their corresponding designated wells in triplicate. Doxorubicin hydrochloride (Pfizer Laboratories, South Africa) was used as positive control. Two wells served as negative controls, thus received no treatment. The blank wells also received no treatment. After treatment, the plates were incubated for 48 h at 37^{o}C in a 5% CO_2 incubator. After incubation, the MEM was gently aspirated and 200 µL of phosphate buffered saline (PBS, Whitehead Scientific), was then used to wash the cells. Following washing, 200 µL of fresh MEM was added to all the wells. Then, 30 µL of 5 mg/mL MTT (Sigma) dissolved in PBS, was added to all the wells. The plates were further incubated for 4 h at 37^{o}C in a 5% CO_2 incubator. Then the medium was again gently aspirated and 50 µL DMSO was added to dissolve the MTT formazan crystals. The plates were shaken gently for 2 – 3 min on a plate shaker (QG - 9001 microporous Quickshaker, Hinotek) to facilitate dissolving of the formazan crystal. A microplate reader (BioTek Synergy) was used to measure the absorbance immediately at 570 nm wavelength. The effect of the samples on the cells (Vero and bovine dermis cells) were determined by calculating the percentage cell viability of cells. The percentage cell viability was calculated as:

% Cell viability = (Mean absorbance of sample / Mean absorbance of control) * 100

Then using the percentage cell viability of the different concentrations tested, the lethal concentration (LC_{50}) was determined. The LC_{50} of test samples, the concentration that can cause 50% reduction of absorbance, compared to the untreated cells was interpreted as the concentration that can be lethal to 50% of the cells. The selectivity index values were calculated by dividing the cytotoxicity, LC_{50} (in mg/mL), by the MIC values (in mg/mL).

2.7. Anti-quorum sensing assay

2.7.1. Bacterial culture for anti-quorum sensing assay

The anti-quorum sensing activity of eight selected plant extracts with significant to moderate activity against *S. aureus* reference and mastitis clinical isolates was performed by the method described by Ahmad et al. (2015), with modification by miniaturizing into 48-well microplates. Briefly, *Chromobacterium violaceum* was maintained on Luria–Bertani (LB) agar and incubated in LB broth overnight for 18 h at 30°C in an orbital shaker (140 rpm) prior to the assay. The overnight bacterial suspension was diluted with sterile distilled water to achieve an absorbance of 0.1±0.02 OD at a wavelength of 590 nm equivalent to McFarland standard No 0.5. This correlated to approximately 1.5 x 10^8 CFU/mL which was diluted in LB broth to a final working inoculum of 1.5 x 10^6 CFU/mL.

2.7.2. Inhibition of violacein production using broth microdilution

Plant extract stock solutions in DMSO (50 mg/mL) were first prepared for each extract. From the 50 mg/mL plant extract stock solution, 50 µL was diluted with 950 µL of LB broth to obtain 2.5 mg/mL solutions. The positive control, gentamicin, was prepared in LB broth to a concentration of 500 µg/mL concentration. *Chromobacterium violaceum* culture (500 µL) was added to wells of 48 well plates except for those wells designated for blank (sample blank and positive control blank). From 2.5 mg/mL samples of plant extracts, 500 µL was added to wells of the wells designated for extract samples. Also 500 µL was taken from the 500 µg/mL concentration of gentamicin and added to the first well of wells designated for the positive control. Extract sample wells and positive control wells were then serially diluted by pipetting 500 µL from the first well, diluting down to the last well and discarding the last 500 µL from the last wells. After the serial dilutions the well concentrations of the samples ranged from 1.25 to 0.009 mg/mL whereas the well concentrations of the positive control ranged from 250 to 1.95 µg/mL. Then 500 µL of LB broth were added to all wells. The blank wells contained 1000 µL of media alone. All wells had a total volume of 1000 µL. The plates were covered with parafilm and incubated for 24 h in an orbital shaker (140 rpm) at 30°C.

After incubation, the anti-quorum sensing activity was determined based on growth (turbidity) and pigmentation (purple colour). Thus, the minimum inhibitory concentration (MIC) value was defined as the lowest concentration of a sample that inhibited the visible growth of *C.*

violaceum, while the minimum quorum sensing inhibitory concentration (MQSIC) values was defined as the lowest concentration of a sample that inhibited the visible pigmentation.

2.7.3. Violacein quantification

After 24 h of incubation of the treated *C. violaceum* culture, 1 mL from each well was transferred to new replica 48 well plates. The plates were centrifuged at 1800 x g (EBA Hettich centrifuge, Labotec) for 20 min to pellet the bacteria containing violacein. The resulting supernatant was discarded and 1 mL of 100% DMSO was added to the bacterial pellet remaining at the bottom of each well. The plates were vortexed until the pellet solubilised. Then the plates were further centrifuged at 1800 x g for 20 min to separate bacterial cells from the solution. Supernatant from each well (200 µL) was transferred to wells of 96-well microtitre plates in duplicate. The absorbance was measured at a wavelength of 595 nm. The percentage violacein inhibition was determined using the formula:

$$\text{Percentage violacein inhibition} = \frac{\text{meanOD}_{control} - \text{meanOD}_{test}}{\text{MeanOD}_{control}} \times 100$$

The concentration of each sample inhibiting 50% of violacein production was determined as the $MQSIC_{50}$.

2.8. Bacterial culture preparation and biofilm inhibition assay

Staphylococcus aureus ATCC 29213, *S. epidermidis* ATCC 35984 and two *S. aureus* isolates from clinical mastitis cases (STA 4 and STA 6) were used for determining inhibition of bacterial biofilm. Tryptone Soy agar (TSA) plates were prepared and used for overnight culture of strains at 37°C without shaking.

The method of O'Toole and Kolter (1998) modified by Sandasi et al. (2010) was used for the biofilm inhibition bioassay. Freshly prepared Tryptic Soy broth (TSB) broth (10 mL) in 50 mL sterile tubes were inoculated with bacteria from the Tryptic Soy agar (TS agar) plates and incubated overnight for 18 h at 37°C with shaking. The overnight cultures were standardized by diluting the cultures with TSB to optical density of 0.02 at 590 nm wavelength using a spectrophotometer (Epoch microplate reader: BioTek, United States) to obtain 1.0 x 10^6 CFU/mL (OD_{590} = 0.02 or 1.0 x 10^6 CFU/mL). One hundred µL of the standardised bacterial cultures were added to wells of individually labelled flat bottomed 96 well plates (Lasec, South

Africa) except wells allocated for blanks and positive control. Three biofilm stages were investigated. Plates for biofilm stage 1, i.e., time zero (0 h, effect on bacterial adhesion) were treated immediately. Plates for biofilm stage 2, i.e., that were to be treated at 24 h (effect on pre-formed biofilm), and plates for biofilm stage 3, i.e., 48 h (effect on matured biofilm), were incubated until their respective time of treatment.

Plant extracts used for the treatments were selected based on their MIC activity, level of cytotoxicity as well as their anti-quorum sensing activity. Stock solutions (2 mg/mL) of the plant extracts were prepared in 50% acetone. Treatment was done by adding 100 µL of 2 mg/mL plant extract in twelve replicates to the corresponding labelled well, giving a final concentration of 1 mg/mL in the well. Gentamicin (Virbac, South Africa) was used as positive control, and 100 µL of 2 mg/mL stock solution was added to the corresponding wells to give a final concentration of 1 mg/mL. Acetone in sterile distilled water (50%) was used as negative control. Each well had a final volume of 200 µL. The biofilm experiments were repeated three times. After treatment, plates were sealed with parafilm and incubated for another 24 h at 37^{o}C without shaking.

2.8.1. Assessment of biofilm inhibition with crystal violet (CV) staining assay

The modified method described by Sandasi et al. (2010) was used for the crystal violet (CV) staining assay. After 24 h incubation, plates were washed four times by gently pouring sterile distilled water from the edges to remove unattached cells. The plates were air dried by gently turning them over clean tissue paper, followed by oven drying at 60^{o}C for 45 min. After drying, 100 µL of 0.1% crystal violet was added to all wells to stain adherent cells and the plates were covered and incubated for 15 min in the dark at room temperature. Then the plates were washed again 4 times with sterile distilled water to remove unabsorbed stain. Purple rings indicating biofilms could be observed at this stage at the sides of the wells. Ethanol (150 µL of 100%) was added to all the wells to de-stain the wells and resolubilise the crystal violet bound to the adherent cells. The de-stained solution (100 µL) was transferred into new sterile plates and the absorbance was measured at 590 nm. The mean optical density (OD) of each sample was obtained and the percentage inhibition was determined using the equation below:

$$\text{Percentage inhibition} = \frac{\text{meanOD}_{\text{untreated}} - \text{meanOD}_{\text{treated}}}{\text{MeanOD}_{\text{untreated}}} \times 100$$

2.9. Statistical analysis

The data are presented as the mean ± standard deviation (SD) of three independent experiments. Statistical differences between LC_{50} values of extract and doxorubicin were assessed by two-way ANOVA followed by Dunnett's test using GraphPad Prism 8.

3. Results and Discussion

3.1. Antimicrobial susceptibility

Three of the isolates, STA 1, STA 2 and STA 6 were resistant to two antibiotics (Table 2). The reference *S. aureus* ATCC 29213 strain was only resistant to one antibiotic. The antibiotics to which these organisms were resistant to fell into two classes of antibiotics, namely the penicillins and the aminoglycosides. All the tested *S. aureus* strains were resistant to ampicillin, suggesting that they could have acquired the *mecA* gene or its homologue, the *mecC* gene. The *mecA* gene encodes for the penicillin-binding protein (PBP2') which has a very low binding affinity for β-lactam antibiotics (Saga and Yamaguchi, 2009). Alternatively, the resistant strains may have the ability to produce the β-lactamase enzyme that inactivates β-lactam antibiotics by hydrolysing the β-lactam ring (Pexara et al., 2013). In addition to being resistant to ampicillin, STA 1, STA 2 and STA 6 were also resistant to amikacin. Amikacin binds to the 30s ribosomal subunit to exert activity. However, it has been found that some *S. aureus* develop resistance to amikacin via an adaptive mechanism through cell wall thickening (Yuan et al., 2013). Thus, the strains STA 1, STA 2 and STA 6 may have developed cell wall thickening and subsequent resistance to amikacin.

Table 2. Diameter of inhibition zones (in mm) of antibiotics against *Staphylococcus aureus* strains and susceptibility interpretation

	***S. aureus* ATCC 29213**		**STA 1**		**STA 2**		**STA 3**		**STA 4**		**STA 5**		**STA 6**	
Antibiotics	**D. I. (mm)**	**Interp.**	**D. I. (mm)**	**Interp.**	**D. I. (mm)**	**Interp.**	**D. I. (mm)**	**Interp.**	**D. I. (mm)**	**Interp.**	**D. I. (mm)**	**Interp.**	**D. I. (mm)**	**Interp.**
Ampicillin (10 µg)	25.60	**R**	23.00	**R**	24.24	**R**	22.68	**R**	25.44	**R**	25.40	**R**	22.20	**R**
Amoxicillin/Clavulanic acid (30 µg)	26.60	S	25.76	S	28.12	S	25.20	S	24.80	S	27.20	S	27.00	S
Ceftiofur (30 µg)	25.05	S	25.40	S	24.04	S	26.20	S	27.20	S	24.12	S	27.2	S
Doxycycline (30 µg)	38.00	S	28.04	S	31.40	S	34.06	S	26.56	S	28.56	S	35.48	S
Sulphamethoxazole /Trimethoprim (25 µg)	30.32	S	26.08	S	27.40	S	29.06	S	27.60	S	26.6	S	26.2	S
Tetracycline (30 µg)	34.92	S	25.00	S	28.44	S	33.02	S	28.40	S	28.00	S	35.64	S
Chloramphenicol (30 µg)	23.76	S	18.04	S	25.00	S	26.08	S	23.80	S	24.44	S	25.60	S
Amikacin (30 µg)	20.72	S	12.80	**R**	14.60	**R**	18.34	S	16.08	I	16.80	I	10.00	**R**

ATCC = *S. aureus* wild type, STA 1-6 = STA 1 to 6 = *S. aureus* mastitis clinical isolates, D. I. = Diameter of Inhibition, Interp. = Interpretation, **R** = Resistant, S = Susceptible, I = Intermediate.

3.2. Plant extract yield, minimum inhibitory concentration and total antibacterial activity

For all the plants, ethanol extracts had the highest extract yield compared to acetone extracts (Table 3). *Elaeodendron croceum* ethanol extract had the highest extract yield of 271.05 mg (27.11%), followed by *K. gunniae* leaf ethanol extract with extract yield of 260 mg (26%). *Maurocenia frangula* acetone extract, *M. undata* acetone extract and *K. gunniae* leaf extracts had promising activity against *S. aureus* ATCC strain with MIC values ranging from 0.02 to 0.08 mg/mL (Table 3). MIC values above 1 mg/mL are considered inactive (Holetz et al., 2002), MIC values between 0.6 mg/mL and 1 mg/mL are considered to be weak activity (Aligiannis et al., 2001; Holetz et al., 2002), MIC values between 0.1 mg/mL and 0.5 mg/mL were regarded as moderate activity (Holetz et al., 2002), while MIC values less than 0.1 mg/mL are regarded as having very good activity (Kuete, 2010). Several plant extracts had moderate activity against *S. aureus* ATCC strain with MIC values ranging from 0.12 mg/mL to 0.47 mg/mL. Six plant extracts had weak activity against *S. aureus* ATCC strain with MIC of 0.63 mg/mL and six other plant extracts were inactive with MIC values ranging from 1.25 mg/mL to > 2.5 mg/mL.

The total antibacterial activity of all the plant extracts against *S. aureus* ATCC 29213 ranged from 6.12 mL/g to 3250.00 mL/g (Table 3). *K. gunniae* leaf acetone extract had the highest total antibacterial activity (3250.00 mL/g) against *S. aureus* followed by *M. frangula* acetone extract with total antibacterial activity (2292.50 mL/g). The TAA indicates the volume to which an extract prepared from 1 g of a plant material can be diluted and still have activity, thus the higher the TAA the better (Eloff, 2004; Dzoyem et al., 2014).

Maurocenia frangula, Maytenus undata, *Kalanchoe gunniae* and *Bryophyllum pinnatum* had the best activities against *S. aureus* ATCC 29213. Therefore, the acetone and ethanol extracts of these species were further assayed for antimicrobial activity against the six *S. aureus* mastitis clinical isolates.

Table 3. Extraction yield, MIC (mg/mL) and TAA (mL/g) of plant extracts against *S. aureus* ATCC 29213

Plant species	Solvent	Yield (mg)	Yield (%)	MIC	TAA
G. heterophylla	Acetone	45.45	4.55	0.63	72.14
	Ethanol	96.95	9.70	0.63	153.89
E. transvaalense	Acetone	37.85	3.79	0.16	236.56
	Ethanol	53.93	5.39	0.31	173.95
G. senegalensis	Acetone	36.88	3.69	0.63	58.53
	Ethanol	102.45	10.25	0.16	640.31
E. croceum	Acetone	63.78	6.38	0.16	398.59
	Ethanol	271.05	27.11	0.16	1694.06
Ilex mitis leaf	Acetone	25.00	2.50	0.63	39.68
	Ethanol	137.50	13.75	0.63	218.25
Ilex mitis stem-bark	Acetone	11.63	1.16	1.50	7.75
	Ethanol	32.93	3.30	2.50	13.17
M. frangula	Acetone	45.85	4.59	**0.02**	2292.50
	Ethanol	68.98	6.90	0.31	222.50
M. peduncularis	Acetone	51.95	5.20	0.63	82.46
	Ethanol	70.20	7.02	0.31	226.45
R. eucleiforme	Acetone	51.123	5.11	0.63	81.15
	Ethanol	83.83	8.38	0.31	270.40
P. streyi	Acetone	18.43	1.84	0.31	59.44
	Ethanol	38.30	3.83	0.16	239.38
C. edulis	Acetone	27.50	2.75	1.25	22.00
	Ethanol	130.00	13.00	0.16	812.50
G. buxifolia	Acetone	30.00	3.00	0.47	63.83
	Ethanol	92.50	9.25	1.25	74.00
M. aethiopicum	Acetone	37.50	3.75	0.63	59.52
	Ethanol	85.00	8.50	1.25	68.00
M. undata	Acetone	77.50	7.75	**0.08**	968.75
	Ethanol	110.00	11.00	0.12	916.67
K. gunniae leaf	Acetone	162.50	16.25	**0.08**	2031.25
	Ethanol	260.00	26.00	**0.08**	3250.00
B. pinnatum leaf	Acetone	30.00	3.00	0.31	96.77
	Ethanol	45.00	4.50	0.31	145.16
K. gunniae stem-bark	Acetone	62.50	6.25	0.16	390.63
	Ethanol	170.00	17.00	0.16	1062.50
B. pinnatum stem-bark	Acetone	15.30	1.53	> 2.5	6.12
	Ethanol	22.50	2.25	1.25	18.00
Gentamicin		NA		0.0002	NA

Values in bold indicate significant antibacterial activity (Eloff, 2021)

M. frangula (MIC = 0.02 mg/mL) and *K. gunniae* (MIC = 0.04 mg/mL) acetone extracts had significant activities against all the *S. aureus* mastitis clinical isolates (Table 4). *Maytenus*

undata acetone extract had significant activity with MIC ranging from 0.04 to 0.08 mg/mL against all the *S. aureus* isolates, with the exception of STA 3, against which it exhibited moderate activity (MIC = 0.11 mg/mL). In terms of the ethanol extracts, both *M. frangula* (MIC = 0.02 - 0.04 mg/mL) and *K. gunniae* (MIC = 0.04-0.08 mg/mL) had significant activity against all the *S. aureus* isolates from clinical mastitis. In general, acetone extracts of *M. frangula* and *K. gunniae* had significantly better activity than ethanol extracts against all the *S. aureus* clinical isolates. This suggests that acetone extracts should be considered for further research on antimicrobial activities of the two plants. Worth noting is that this is the first study to report on the promising antibacterial activity of *Kalanchoe gunniae,* a newly described nothospecies in South Africa (Smith et al., 2019).

Similar to the acetone extract, *M. undata* ethanol extract had significant activity with MIC ranging from 0.04 to 0.08 mg/mL against five of the *S. aureus* isolates from clinical mastitis (STA 1, STA 2, STA 4, STA 5, and STA 6) and moderate activity of 0.11 mg/mL against one of the clinical isolates (STA 3). *Bryophyllum pinnatum* acetone extract had significant activity with MIC of 0.08 mg/mL against four of the *S. aureus* clinical isolates (STA 1, STA 2, STA 3, STA 4), moderate activity of 0.16 mg/mL against STA 5, and weak activity of 0.63 mg/mL against STA 6. The ethanol extract of *B. pinnatum* had moderate activity (MIC = 0.16 -0.32 mg/mL) against all the *S. aureus* clinical isolates. The results also suggest that the acetone extract of *B. pinnatum* was more active than the ethanol extract against the *S. aureus* clinical isolates. The findings of this study are similar to the findings by Akinsulire et al. (2007) which reported that the methanol extract of *B. pinnatum* had good antimicrobial activity against *S. aureus*.

The mean MIC of the eight plant extracts against all seven strains ranged from 0.0200 mg/mL to 0.2600 mg/mL (Table 4). Among acetone extracts, *M. frangula* acetone extract had the most significant mean MIC (0.0200 mg/mL) against all seven bacterial strains followed by *K. gunniae* acetone extract (0.0457 mg/mL). Among the ethanol extracts, *K. gunniae* extract had the most significant mean MIC (0.0629 mg/mL) against all seven bacterial strains followed by *M. frangula* (0.0643 mg/mL).

Of the acetone extracts, *K. gunniae* leaf acetone extract had the highest total antibacterial activity (4062.50 mL/g) against all *S. aureus* mastitis clinical isolates followed by *M. frangula* acetone extract (2292.50 mL/g), also against all *S. aureus* mastitis clinical isolates. Total antibacterial activity of the extracts against *S. aureus* mastitis clinical isolates ranged from 47.62 mL/g to 6500.0 mL/g (Table 4). From the ethanol extracts, *K. gunniae* leaf ethanol extract had the highest total antibacterial activity against *S. aureus* mastitis clinical isolates ranging from 3250.00 mL/g to 6500.00, followed by *M. frangula* acetone extract with total antibacterial activity ranging from 1724.38 mL/g to 3448.75 mL/g. The ethanol extract of *K. gunniae* which had the highest total activity is a promising candidate for isolation of bioactive compounds, as well as for development into a herbal formulation.

Table 4. MIC (mg/mL) and TAA (mL/g) of the most active plant extracts against *Staphylococcus aureus* ATCC 29213 and clinical isolates STA1 to STA6.

Plant extracts	Extract yield (mg)	% Extract yield	ATCC 29213		STA 1		STA 2		STA 3		STA 4		STA 5		STA 6		Average MIC (mg/mL)
			MIC	TAA	MIC	TAA	MIC	TAA	MIC	TAA	MIC	TAA	MIC	TAA	MIC	TAA	
Acetone																	
MF	45.85	4.59	0.02	2292.5	0.02	2292.50	0.02	2292.50	0.02	2292.50	0.02	2292.50	0.02	2292.50	0.02	2292.50	0.0200
MU	77.50	7.75	0.08	968.75	0.08	968.75	0.08	968.75	0.11	704.55	0.08	3448.75	0.08	968.75	0.04	1937.50	0.0786
KP	162.50	16.25	0.08	2031.25	0.04	4062.5	0.04	4062.5	0.04	4062.5	0.04	4062.5	0.04	4062.50	0.04	4062.50	0.0457
BP	30.00	3.00	0.32	93.75	0.08	375.00	0.08	375.0	0.08	375.00	0.08	375.00	0.16	187.50	0.63	47.62	0.2043
PC	NA		0.0002	NA	0.0006	NA	0.0002	NA	0.0006	NA	0.0002	NA	0.0002	NA	0.0008	NA	0.0004
Ethanol																	
MF	68.98	6.90	0.31	222.50	0.02	3448.75	0.04	1724.38	0.02	3448.75	0.02	3448.75	0.02	3448.75	0.02	3448.75	0.0643
MU	110.00	11.00	0.12	916.67	0.08	1375.0	0.08	1375.00	0.11	1000.00	0.08	968.75	0.08	1375.00	0.04	2750.00	0.0843
KP	260.00	26.00	0.08	3250.00	0.08	3250.0	0.04	6500.00	0.08	3250.00	0.08	3250.00	0.04	6500.00	0.04	6500.00	0.0629
BP	45.00	4.50	0.32	140.63	0.16	281.25	0.27	166.67	0.32	140.63	0.27	166.67	0.16	281.25	0.32	140.63	0.2600
PC	NA		0.0002	NA	0.0006	NA	0.0002	NA	0.0006	NA	0.0002	NA	0.0002	NA	0.0008	NA	

Staphylococcus aureus isolates: STA 1, STA 2, STA 3, STA 4, STA 5 and STA 6. MF = *Maurocenia frangula*, MU = *Maytenus undata*, KP = *Kalanchoe gunniae*, BP = *Bryophyllum pinnatum*, PC = (Positive control) Gentamicin, N.A = Not applicable. Values in bold indicate significant antibacterial activity (Eloff, 2021)

3.3. Cytotoxicity

Extracts of *K. gunniae* and *B. pinnatum* were least cytotoxic to Vero and bovine dermis cells, while extracts of *M. undata* were most toxic (Table 5). Cytotoxicity investigation helps to ascertain whether the activity observed with a plant extract is as a result of toxicity (Elisha et al., 2017). According to Kuete (2010), a plant extract should be considered toxic when the lethal concentration killing 50% of cells (LC_{50}) is 0.02 mg/mL and below. None of the plant extracts tested had LC_{50} value below 0.02 mg/mL (Table 5).

The MIC values for extracts reported in Table 4 were used to calculate selective index (SI) values in an effort to identify extracts with better (selective) activity against *S. aureus* relative to toxicity against mammalian cells (Vero and bovine dermis). *K. gunniae* extracts had the best SI values for both Vero cells and for bovine dermis cells for all tested organisms, followed by *B. pinnatum* (Table 5). Acetone and ethanol extracts of *M. frangula* in all cases had SI values greater than one for Vero cells. However, the ethanol extract had SI values greater than one for bovine dermis cells with the clinical isolates and less than one with the ATCC strain. Of interest is that, although *M. frangula* extracts have low LC_{50} values, the much lower MIC values obtained for its acetone extract (Table 4, MIC = 0.02 mg/mL) against all strains and its ethanol extract against the clinical isolates, increased the corresponding SI values.

Extracts of *K. gunniae*, *M. frangula* and *B. pinnatum* had average SI values >1 for Vero and bovine dermis cells for all seven *S. aureus* strains (Table 6). *Kalanchoe gunniae* extracts had the highest average SI values followed by *M. frangula* extracts. The average SI of acetone extracts of *K. gunniae* were 20 and 31.80 for Vero and bovine dermis cells respectively, which is highly promising. *K. gunniae* ethanol extract also had a high average SI of 16.00 and 16.67 for Vero and bovine dermis cells respectively. The average SI of *M. frangula* acetone extract was 13.50 (Vero) and 7.50 (bovine dermis) cells and the ethanol extract had average SI values of 7.85 (Vero) and 3.75 (bovine dermis) cells.

SI values above 1 indicate greater activity against bacteria than toxicity to mammalian cells (Sserunkuma et al., 2017). The higher the SI value of a plant extract, the better and the safer it is to develop herbal therapeutic formulations from such plant extracts (Dzoyem et al., 2014). Thus, *K. gunniae* extracts which had the highest average SI values hold much value for the

future development of herbal therapeutic formulations for the treatment of clinical mastitis associated with *S. aureus.*

Table 5. LC_{50} of most active extracts on Vero and bovine dermis (BD) cells and their selective index values on *S. aureus* isolates from clinical mastitis

Plant extracts	Cell line	LC_{50} (mg/mL)	Selectivity index						
			ATCC	STA 1	STA 2	STA 3	STA 4	STA 5	STA 6
MFa	Vero	0.27±0.05****	13.5	13.5	13.5	13.5	13.5	13.5	13.5
	BD	0.15±0.02*	7.5	7.5	7.5	7.5	7.5	7.5	7.5
MFe	Vero	0.50±0.06****	1.61	25	12.5	25	25	25	25
	BD	0.24±0.02***	0.77	12	6	12	12	12	12
MUa	Vero	0.05±0.007	0.63	0.63	0.63	0.46	0.63	0.63	1.25
	BD	0.12±0.005	1.50	1.50	1.50	1.09	1.50	1.50	3.0
MUe	Vero	0.06±0.009	0.5	0.75	0.75	0.55	0.75	0.75	1.5
	BD	0.23±0.04***	1.92	2.88	2.88	0.55	2.88	2.88	5.75
KPa	Vero	>1.0±0.10****	**12.50**	**25.0**	**25.0**	**25.0**	**25.0**	**25.0**	**25.0**
	BD	>1.0±0.09****	**12.50**	**25.0**	**25.0**	**25.0**	**25.0**	**25.0**	**25.0**
KPe	Vero	0.96±0.05****	**12**	**12**	**24**	**12**	**12**	**24**	**24**
	BD	>1.0±0.12****	**12.50**	**12.50**	**25.0**	**12.50**	**12.50**	**25.0**	**25.0**
BPa	Vero	0.86±0.01****	2.69	10.75	10.75	10.75	10.75	5.38	1.37
	BD	0.62±0.05****	1.94	7.75	7.75	7.75	7.75	3.88	1.94
BPe	Vero	>1.0±0.14****	3.13	6.25	3.70	3.13	3.70	6.25	3.13
	BD	0.46±0.06****	1.44	2.88	1.70	1.44	1.70	2.88	1.44
Doxorubicin	Vero	0.0099 ± 0.001327							
	BD	0.0087 ± 0.0008							

MFa = *Maurocenia frangula* acetone extract, MFe= *Maurocenia frangula* ethanol extract, Ma = *Maytenus undata* acetone extract, MUe = *Maytenus undata* ethanol extract. KPa = *Kalanchoe gunniae* acetone extract, KPe = *Kalanchoe gunniae* ethanol extract, BPa = *Bryophyllum pinnatum* acetone extract, BPe = *Bryophyllum pinnatum* ethanol extract. BD = bovine dermis cells, ATCC = *S. aureus* ATCC 29213. STA 1, STA 2, STA 3, STA 4, STA 5 and STA 6 are *S. aureus* clinical isolates, Statistical analysis was performed with Dunnett's multiple comparisons test using two-way ANOVA; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$ between extracts and doxorubicin.

Table 6. Mean MIC and average selective indexes of the most active plant extracts on all seven strains (clinical isolates and *S. aureus* ATCC 29213)

								Mean MIC (mg/mL)	Vero cells		Bovine dermis cells	
Samples	**ATCC**	**STA 1**	**STA 2**	**STA 3**	**STA 4**	**STA 5**	**STA 6**		**LC_{50} (mg/mL)**	**Average SI**	**LC_{50} (mg/mL)**	**Average SI**
Acetone extracts												
MF	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.27	13.5	0.15	7.5
MU	0.08	0.08	0.08	0.11	0.08	0.08	0.04	0.08	0.05	0.63	0.12	1.5
KP	0.08	0.04	0.04	0.04	0.04	0.04	0.04	0.05	>1.0	20.0	>1.0	20.0
BP	0.32	0.08	0.08	0.08	0.08	0.16	0.63	0.20	0.86	4.3	0.62	3.1
Gentamicin	0.0002	0.0006	0.0002	0.0006	0.0002	0.0002	0.0008	0.0004	NA	NA	NA	NA
Ethanol extracts												
MF	0.31	0.02	0.04	0.02	0.02	0.02	0.02	0.064	0.50	7.81	0.24	3.75
MU	0.12	0.08	0.08	0.11	0.08	0.08	0.04	0.08	0.06	0.75	0.23	2.88
KP	0.08	0.08	0.04	0.08	0.08	0.04	0.04	0.06	0.96	16.0	>1.0	16.67
BP	0.32	0.16	0.27	0.32	0.27	0.16	0.32	0.26	>1.0	3.85	0.46	1.77
Gentamicin	0.0002	0.0006	0.0002	0.0006	0.0002	0.0002	0.0008	0,0004	NA	NA	NA	NA

MF = *Maurocenia frangula*, MU = *Maytenus undata*, KP = *Kalanchoe gunniae*, BP = *Bryophyllum pinnatum*, NA = Not applicable. ATCC = *S. aureus* ATCC 29213. STA 1, STA 2, STA 3, STA 4, STA 5 and STA 6 are *S. aureus* clinical isolates

3.4. Quorum sensing

Minimum quorum sensing inhibition concentration (MQSIC) of the tested extracts ranged from 0.04 to 0.63 mg/mL and their MIC values ranged from 0.63 to 1.25 mg/mL (Table 7). *Kalanchoe gunniae* extracts had the best anti-quorum sensing activity with strong MQSIC of 0.04 mg/mL and weak MIC value of 0.63 mg/mL indicating that the MQSIC concentration was not toxic to the bacteria. Both extracts of *B. pinnatum*, the ethanol extract of *M. frangula* and ethanol extract of *M. undata* had anti-quorum sensing activity with moderate MQSIC of 0.16 mg/mL and inactive MIC value of either 1.25 or >1.25 mg/mL indicating that the MQSIC concentration did not prevent bacterial growth. *Maytenus undata* acetone extract had poor anti-quorum sensing activity with weak MQSIC activity of 0.63 mg/mL and inactive MIC of > 1.25 mg/mL indicating that the extract neither inhibited quorum sensing nor killed or reduced growth of bacterial cells.

Table 7. Minimum quorum sensing inhibition concentration (MQSIC), minimum inhibitory concentration (MIC) of the most active extracts against *C. violaceum* and their $MQSIC_{50}$ (50% inhibitory concentration).

Plant/Samples	Solvent (extract)	MQSIC (mg/mL)	MIC (mg/mL)	$MQSIC_{50}$ (mg/mL)
M. frangula	Acetone	0.31	> 1.25	0.95
	Ethanol	0.16	1.25	0.87
M. undata	Acetone	0.63	> 1.25	0.79
	Ethanol	0.16	1.25	0.63
K. gunniae	Acetone	0.04	0.63	0.08
	Ethanol	0.04	0.63	0.07
B. pinnatum	Acetone	0.16	1.25	0.67
	Ethanol	0.16	1.25	0.57
Gentamicin		0.008	0.008	0.99

In *C. violaceum*, violacein pigment production is mediated by acylated homoserine lactone (AHL). Quorum sensing mechanism of action can be quantified by targeting the autoinducer, AHL mediated functions. Therefore, violacein production or inhibition of *C. violaceum* in culture media can be used to measure the QS mechanism (Vattem et al., 2007). The minimum quorum sensing inhibition concentration that inhibited 50% of violacein production ($MQSIC_{50}$) of the extracts against *C. violaceum* ranged from 0.07 mg/mL to 0.95 mg/mL (Table 7). *Kalanchoe gunniae* extracts had the best $MQSIC_{50}$ with values for ethanol and acetone extracts

of 0.07 mg/mL and 0.08 mg/mL respectively. These results indicate that both extracts greatly inhibited quorum sensing, without being toxic to the bacteria.

The quorum sensing selectivity index (quorum sensing SI) of the plant extracts against Vero and bovine dermis cells was also calculated as it is useful quantitative way to highlight extracts with good anti-quorum sensing activity without being toxic to the bacteria or mammalian cells. Extracts of *K. gunniae* and *B. pinnatum* had the highest quorum sensing SI with values greater than 1 with all the extracts (Table 8). The quorum sensing SI of *K. gunniae* extracts ranged from 24.0 to 25.0 for both Vero and bovine dermis cells with mean quorum sensing SI values of 25.0 (Vero and BD cells) and 24.5 (Vero and BD cells) for the acetone and ethanol extracts respectively. For *B. pinnatum* extracts, quorum sensing SI ranged from 2.88 to 6.25 for both Vero and bovine dermis cells with mean quorum sensing SI values of 4.63 and 4.57 for acetone and ethanol extracts respectively.

Vasavi et al. (2014) reported that a plant extract fraction containing flavonoids was able to result in significant anti-QS activity. The extracts of *K. gunniae* and *B. pinnatum,* which had significant to good anti-QS activity respectively, most likely have varying degrees of flavonoids or other particular plant secondary metabolite(s) that have activity against bacterial quorum sensing. Further work is necessary to isolate and characterize the active compounds.

Table 8. Selectivity index values of the most active extracts against *Chromobacterium violaceum* using their MQSIC

Samples		LC_{50} (mg/mL)	QSSI	Mean QSSI	MQSIC (mg/mL)
Acetone extracts					
MF	Vero cells	0.27±0.05	0.87	0.68	0.31
	BD cells	0.15±0.02	0.48		
MU	Vero cells	0.05±0.007	0.08	0.14	0.63
	BD cells	0.12±0.005	0.19		
KP	Vero cells	>1.0±0.10	25.0	25.0	0.04
	BD cells	>1.0±0.09	25.0		
BP	Vero cells	0.86±0.01	5.38	4.63	0.16
	BD cells	0.62±0.05	3.88		
Ethanol extracts					
MF	Vero cells	0.50±0.06	3.13	2.32	0.16
	BD cells	0.24±0.02	1.5		
MU	Vero cells	0.06±0.009	0.38	0.91	0.16
	BD cells	0.23±0.04	1.44		
KP	Vero cells	0.96±0.05	24.0	24.5	0.04
	BD cells	>1.0±0.12	25.0		
BP	Vero cells	>1.0±0.14	6.25	4.57	0.16
	BD cells	0.46±0.06	2.88		

MF = *Maurocenia frangula*, MU = *Maytenus undata*, KP = *Kalanchoe gunniae*, BP = *Bryophyllum pinnatum*. QSSI = Quorum sensing selectivity index, Mean QSSI = Mean quorum sensing selectivity index, MQSIC = Minimum quorum sensing inhibition concentration.

3.5. Effect of selected extracts on the inhibition of biofilm

Considering results of the antibacterial activity, cytotoxicity and anti-quorum sensing assays, extracts of *K. gunniae* and *B. pinnatum* were selected for investigation of their biofilm inhibition ability. The anti-biofilm assay was performed against four strains (*S. aureus* ATTC 29213, *S. epidermidis* ATCC 35984, and two of the *S. aureus* isolates, namely STA 4 and STA 6). *Staphylococcus aureus* ATTC 29213 was chosen to represent a reference strain of*S. aureus*, while *S. epidermidis* ATCC 35984 was chosen because it is a known biofilm producer. Based on the results of the antibiogram assay, STA 4 and STA 6 were chosen to represent *S. aureus* mastitis clinical isolates. The biofilm assay was done to check the effect of the extracts on prevention of cell attachment (anti-adhesion) – 0 h, inhibition of development of pre-formed biofilms – 24 h and evaluation of destruction of matured biofilm –48 h.

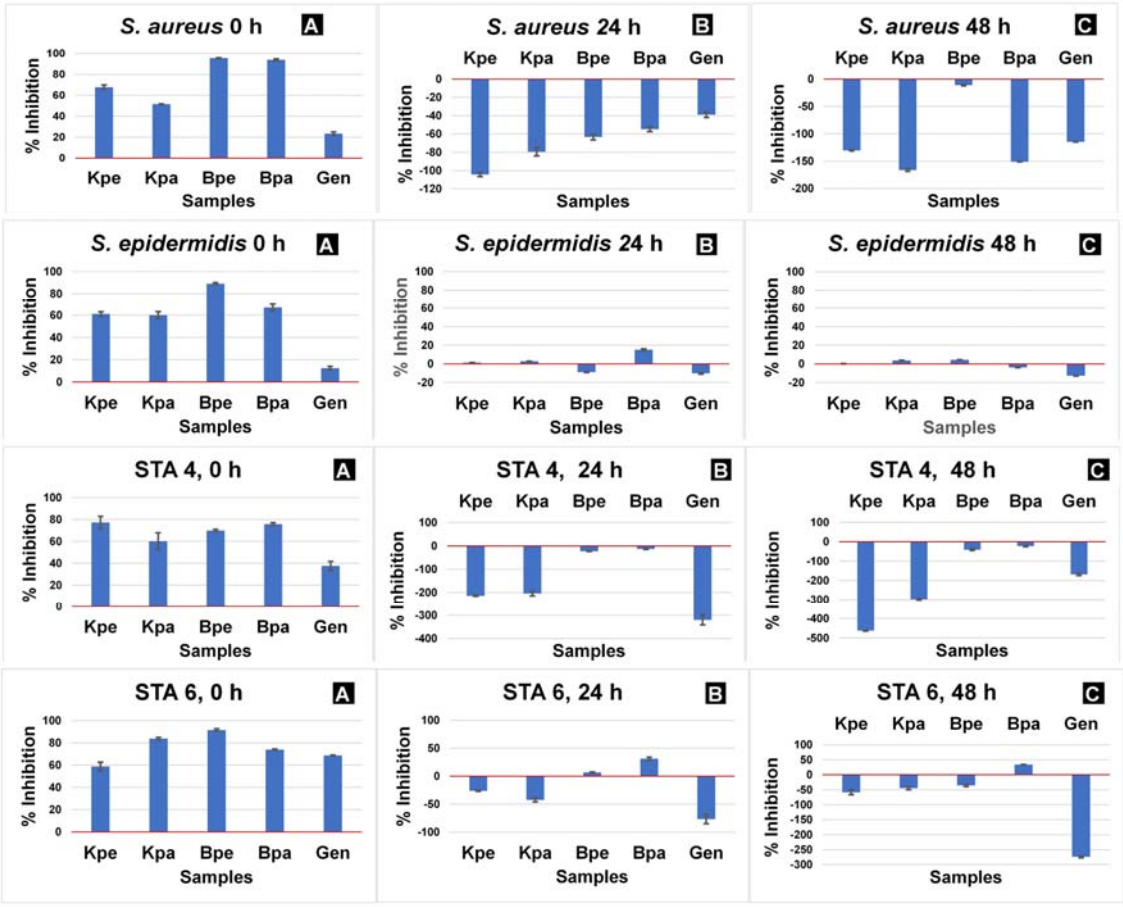

Figure 1. Percentage (%) of biofilm inhibition of plant extracts on *S. aureus* ATCC 29213, *S. epidermidis* ATCC 35984, STA 4 and STA 6. A = Treatment at 0 h (bacterial adhesion stage), B = Treatment after 24 h (pre-formed biofilm stage), C = Treatment after 48 h (matured biofilm stage). Kpa = *Kalanchoe gunniae* acetone extract, Kpe = *Kalanchoe gunniae* ethanol extract, Bpa = *Bryophyllum pinnatum* acetone extract, Bpe = *Bryophyllum pinnatum* ethanol extract, Gen = Gentamicin.

At time zero (0 h), all the extracts had good biofilm inhibition above 50% against all the tested organisms (Figure 1). *B. pinnatum* ethanol extract had the best biofilm inhibition ranging from 69.95% to 95.55% at time zero. The acetone extract of *B. pinnatum* had the second-best biofilm inhibition activity ranging from 67.00% to 93.89% at time zero. Biofilm inhibition activities of *K. gunniae* extracts ranged from 51.53% to 83.73%. Phytochemicals can interfere with the activities of bacterial receptors which are important in the attachment stage of biofilm formation. This can happen by either directly binding to the bacterial receptors or by downregulating the expression and activities of binding factors in the bacterial cells (Hidalgo

et al., 2011). For example, plant secondary metabolites can bind to bacterial cell walls and inhibit enzyme activity, thus preventing the initial cell attachment and biofilm formation (Tiwari et al., 2011). Therefore, it is possible that the above plant extracts contain phytochemicals with anti-biofilm properties and that they may interfere with the functioning of bacterial signaling receptors.

On 24 h-old pre-formed biofilm, *K. gunniae* acetone and ethanol extracts and *B. pinnatum* acetone extracts had weak antibiofilm activity (1.34% to 15.19%) against *S. epidermidis,* whereas *B. pinnatum* ethanol extract enhanced biofilm development of *S. epidermidis.* All the extracts enhanced biofilm development of *S. aureus* and STA 4 on 24 h pre-formed biofilm (Figure 1). For STA 6, *B. pinnatum* acetone and ethanol extracts had weak antibiofilm activity (31.35% and 6.95% respectively) on 24 h pre-formed biofilm, whereas *K. gunniae* acetone and ethanol extracts enhanced the growth of pre-formed biofilm.

On 48 h matured biofilm, none of the extracts had good antibiofilm eradication activity against any of the tested organisms (Figure 1). However, *K. gunniae* acetone and ethanol extracts and *B. pinnatum* ethanol extracts had weak biofilm destruction activity (0.25% to 4.36%) on *S. epidermidis.* The *Bryophyllum pinnatum* acetone extract enhanced the growth of matured biofilm on *S. epidermidis.* None of the extracts had biofilm inhibition activity on the 48h matured biofilm of *S. aureus* and STA 4. The extracts instead enhanced the growth of these organisms. For STA 6 with 48 h matured biofilm, only the *B. pinnatum* acetone extract was able to have at least a weak biofilm inhibition activity (33.74%). All the other extracts enhanced growth of STA 6 in the 48 h matured biofilm.

Overall, this study revealed that extracts from *Kalanchoe* species had the most potent antibacterial and antibiofilm activity. Our finding is in agreement with the literature since different classes of bioactive secondary metabolites such as bufadienolides, terpenoids and flavonoids have been isolated from *Kalanchoe* species (Costa et al., 2008). Considering the pharmacological potential of flavonoids and bufadienolides, these compounds and other metabolite classes present in *Kalanchoe* species might be responsible for the observed activity.

4. Conclusion

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In this study, extracts of *Kalanchoe gunniae* had excellent antibacterial and antibiofilm activity against resistant *S. aureus from* clinical cases of mastitis. These extracts have potential for development as formulations for the prevention and possible treatment of mastitis. Further work is ongoing to explore the possibilities of *in vivo* studies of herbal therapeutic mastitis spray formulations for both prophylaxis and treatment of bovine mastitis.

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