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In vitro biological activities of *Combretum molle* R.Br. ex G. Don (Combretaceae) against mastitis-causing organisms

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ABSTRACT

Bovine mastitis, caused mostly by *Staphylococcus* species, has gained global importance owing to the increasing prevalence of multi-drug resistant bacteria, which comprise a risk to food security and public health. In this study, the antibacterial, anti-biofilm and quorum quenching activities of six leaf extracts of *Combretum molle* R.Br. ex G. Don prepared using different solvents were assessed against clinical isolates of *Staphylococcus aureus* and two *Staphylococcus* ATCC strains (*S. aureus* ATCC 29213 and *S. epidermidis* ATCC 35984). The antioxidant and anti-inflammatory activity and cytotoxicity of the extracts was also evaluated. The antibacterial and anti-biofilm potential of the extracts was determined via serial microdilution and crystal violet and INT assays. Quorum quenching activity was ascertained via inhibition of violacein production in *Chromobacterium violaceum* ATCC 12472. The antioxidant activity was determined using in vitro chemical assays. The 15-lipoxygenase enzyme inhibition and the nitric oxide inhibition assays were utilized to ascertain the anti-inflammatory activity of the extracts. The tetrazolium-based colorimetric (MTT) reduction assay was used to determine the cytotoxicity of the extracts against Vero African green monkey kidney cells. The antibacterial activity of the extracts was moderate to good, with minimum inhibitory concentration (MIC) values of 0.02–0.63 mg/mL. The extracts had promising biofilm inhibition (≥50 %) against four of the test strains. The effects of the extracts on the metabolic activity of the test strains showed ≥ 50 % inhibition in most of the test strains at the different test times. All extracts had moderate to excellent quorum quenching activity at different concentrations with an inhibition ranging between 32.81- 96.32 %. The methanol and cold water extracts had the best antioxidant activity against the 2, 2-diphenyl-1-picrylhydrazyl (DPPH) and 2, 2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radicals with IC_{50} of 7.79 and 2.34 μg/mL, respectively. The hot water extract (AQH) had the best anti-inflammatory activity in terms of 15-lipoxygenase enzyme inhibition with 85.26 % inhibition, while the methanol extract had the best nitric oxide inhibition in LPS-induced RAW 264.7 macrophages with inhibition of 96.75 % at 100 μg/mL. Selectivity index values of the extracts were as high as 50. *Combretum molle*, therefore, offers promising potential as a source of antibiofilm and anti-quorum sensing agents with the ability to disrupt microbial virulence factors. Additionally, the extract had good antioxidant and anti-inflammatory properties, supporting further research into its use in preventing and treating bovine mastitis.

1. Introduction

Bovine mastitis is an inflammation of the mammary parenchyma and is usually caused by an infection with microorganisms in the udder, or from the environment, which includes manure, dirty stalls, hands of milking personnel and milking equipment (Vasudevan et al., 2003; Alberta Milk, 2019). Mastitis infection can be either clinical, with apparent signs, or subclinical which is asymptomatic, but contributes to vast financial losses in the dairy industry (Petrovski et al., 2006; Viguier et al., 2009). Mastitis, which affects all milk-producing mammals, is a major problem in the dairy sector, with poorly managed mastitis infections resulting in a loss in milk production, reduced milk quality, and in some cases, death (Gemechu et al., 2019).

In South Africa, there has been a decline in milk producers over the past 10 years (Karzis et al., 2018) and this may be associated with the prevalence of mastitis infections affecting productivity

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and management. Between 2002 and 2006 in South Africa, a 7.3 % and 5.9 % increase in subclinical and clinical cases of mastitis respectively was recorded, while the financial loss worldwide resulting from these cases is estimated to be 35 billion USD (Karzis et al., 2018). Banga et al. (2014) reported that in South Africa, the cost due to losses associated with mastitis varies between USD 25.88 to 94.55 per cow per year when the bulk somatic cell count increases by 1 % depending on the cow breed and production system. Somatic cells (SC) in milk comprise a combination of milk-building cells and leucocytes which are present in milk and help in determining the quality of the milk. The increase in the production of SC in milk can reduce the quality of milk (Alhussien and Dang 2018).

Mastitis-causing organisms produce tenacious infections, and *Staphylococcus* and *Streptococcus* species are the most common. *Staphylococcus aureus* is an intracellular bacterial species that often forms abscesses in the udder parenchyma that can lead to chronic infection and could develop drug resistance (Kobayashi et al., 2015; Abera 2020; Pedersen et al., 2021). Most staphylococci isolated from bovine mastitis cases can exist as biofilms by binding to milk fat globule membranes, causing an interaction between the organism and the antigen determinant of the host cell, thereby enhancing the virulence ability of the organism which could lead to antimicrobial resistance (Ali-Vehmas et al., 1997; Sserunkuma et al., 2017; Phophi et al., 2019).

With the current prevalence and increased rate of antibiotic resistance, there is a strong interest in developing an alternative mode of treatment for various animal diseases, and the use of medicinal plants in ethnoveterinary medicine in the treatment of various animal diseases has been reported in different cultures and countries (Mussarat et al., 2014; Chitura et al., 2018; Khunoana et al., 2019; Adeniran et al., 2020; Khan et al., 2021). There has been a concomitant increase in the investigation of traditional medicinal plants for combating drug resistance strains, including plants with antibiofilm and quorum quenching effects (Rani and Khullar 2004; Erhabor et al., 2019; Ugboko et al., 2020; Kebede et al., 2021). Detailed reviews of medicinal plants used in ethnoveterinary medicine in South Africa have been documented (McGaw and Eloff 2008; Maphosa and Masika 2010; McGaw et al., 2020).

Combretum molle belongs to the Combretaceae family, species of which are commonly used in traditional medicine in most African countries. *Combretum molle* has been associated with treating inflammatory conditions, fever, general body swellings and as a dressing for wounds (Anato et al., 2018a, 2018b; Garba et al., 2018; Rademan et al., 2019). *Combretum molle* is also used to treat gastric ulcers, stomach pains and other abdominal disorders (Anato et al., 2018a). It has been used in traditional medicine in Ethiopia in cleaning milking equipment to avoid milk spoilage (Regassa and Araya, 2012). *Combretum molle* has a wide range of pharmacological activities, including anti-inflammatory (McGaw et al., 2001), antibacterial (Regassa and Araya 2012; Mogashoa et al., 2019), antioxidant and anti-proliferative activities (Rademan et al., 2019). *C. molle* was selected in this study based on its traditional uses and its known pharmacological effects. The aim of this study was to determine the antibacterial activity of different solvent extracts of *C. molle* against six *Staphylococcus* species isolated from clinical cases of bovine mastitis as well as two ATCC reference strains. The antibiofilm, quorum quenching, antioxidant, anti-inflammatory, and cytotoxic effects of the extracts were also determined.

2. Materials and methods

2.1. Plant collection and extraction

The leaves of *C. molle* were collected in the Pretoria National Botanical Garden, Pretoria, South Africa. The plant material was cleaned and then dried in a well-ventilated room at room temperature for two to four weeks. A herbarium voucher specimen was prepared and deposited at the H.G.W.J. Schweickerdt Herbarium (PRU 130,494), University of Pretoria, South Africa. The dried plant material was milled to a fine powder and kept in glass jars in the dark until required. The finely ground plant material was extracted (Eloff, 1998a) using solvents of varying polarity. Methanol, ethanol, acetone, dichloromethane: methanol (1:1), and water (cold and hot) were used for extraction in a ratio of 1:10 (w:v) of plant material to solvent. The mixtures were soaked at 25 °C for 48 h and filtered through Whatman No 1 filter paper. The supernatant was poured out into weighed labelled glass vials. The extraction process was repeated twice to fully extract the plant material. The solvent was evaporated in a Büchi Rotavapor (Labotec (Pty) Ltd.) under reduced pressure at 40 °C until left with a residue that was dried completely under a stream of cold air. The percentage yield was calculated using the formula:

$$\text{Percentage yield} = (\text{Mass of extract}/\text{Mass of powdered plant material}) \times 100$$

2.2. Culturing bacterial strains

Mueller Hinton agar (MHA) and Mueller Hinton broth (MHB) were used for the assessment of the minimum inhibitory concentration test while Luria Bertani (LB) broth was used for the anti-quorum sensing assay. Tryptic Soy agar (TSA) and Tryptic Soy Broth (TSB) were used in the biofilm assays respectively. Six clinical isolates of *Staphylococcus aureus* from bovine mastitis cases (Ethics permission number V121–16, University of Pretoria) and two *Staphylococcus* ATCC strains (*S. aureus* ATCC 29213, *S. epidermidis* ATCC 35984) were collected from the Milk Laboratory, Department of Production Animal Studies, Faculty of Veterinary Sciences, University of Pretoria were used in this study. The bacteria were grown in MHA (Fluka, Spain) and kept at 4 °C. The bacterial cultures were cultured in MHB for at least 18–24 h at 37 °C in an incubator.

2.3. Antibiogram assay

The susceptibility and resistance of the bacterial isolates were tested against ten antibiotics using a variation of the disk diffusion method with measured zones of inhibition (Bauer *et al*., 1966). These included beta-lactams (penicillin G 10 IU, ampicillin 10 μg, cloxacillin 5 μg, amoxycillin 30 μg, ceftiofur 30 μg), tetracyclines (oxy-tetracycline 30 μg), cephalosporins (cephalexin 30 μg, cefuroxime 30 μg, cephalothin 30 μg), aminoglycoside (kanamycin 30 μg) and rifampin (40 μg). Standardized bacterial suspensions of the microorganisms equivalent to 0.5 McFarland standard corresponding to 1×10^8 CFU/mL were prepared in normal saline. 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 for 18 to 24 h at 37 °C. The experiment was done on six isolates of *S. aureus*. After incubation, the zones of inhibition were measured according to the performance standard of the Clinical and Laboratory Standards Institute for antimicrobial disk susceptibility tests for bacteria isolated from animals (CLSI, 2020).

2.4. Determination of minimum inhibitory concentration

The minimum inhibitory concentration (MIC) of the extracts was determined following the method of Eloff (1998b). The extracts were tested at a starting concentration of 2.5 mg/mL (from a stock concentration of 10 mg/mL) and the experiment was carried out in triplicate.

2.5. Anti-biofilm assay

2.5.1. Inhibition of biofilm formation

The inhibition of biofilm biomass formation was evaluated via the protocol described by Sandasi et al. (2010). Biofilms were allowed to form for 24 h (T24) and 48 h (T48). After this the crystal violet staining (CVS) assay was performed to assess the biofilm biomass or cell adhesion. The extracts were tested at a final concentration of 1 mg/mL from a stock of 2 mg/mL and the respective controls at 0.5 mg/mL from a stock of 1 mg/mL.

2.5.2. Crystal violet staining assay

Firstly, the wells were carefully emptied and washed at least three times with sterile distilled water to remove any unattached cells. The plates were then oven-dried for 45 min at 65 °C, and the adhered cells were stained with 100 μL of 0.1 % crystal violet solution for 15 min at room temperature while covered with the microtitre lids. The plates were washed at least five times with distilled water to rinse off any excess unabsorbed stains. Afterwards, the biofilm biomass reduction/inhibition was evaluated semi-quantitatively by re-solubilizing the crystal violet stain bound to the adherent cells with 150 μL of 100 % ethanol. The absorbance of the plates after careful shaking was read at 590 nm using a microplate reader (EpochTM Microplate Spectrophotometer). The mean absorbance (OD_{590}) was determined, and results were expressed as percentage inhibition using the equation below (Sandasi et al., 2010).

$$\% \text{ inhibition} = \frac{\left[\left(OD_{\text{negative control}} - OD_{\text{media control}}\right) - \left(OD_{\text{sample}} - OD_{\text{sample control}}\right)\right]}{\left(OD_{\text{negative control}} - OD_{\text{media control}}\right)} \times 100$$

2.5.3. Assessment of biofilm metabolic activity/viability of biofilm cells

The effect of the extracts on bacterial metabolic activity to determine biofilm cell viability was determined following the modified [2-(4-iodophenyl)−3-(4-nitrophenyl)−5-phenyl-2H-tetrazolium] (INT) reduction assay (Sandasi et al., 2010; Klančnik et al., 2014). The INT (Sigma Aldrich) was prepared with sterile distilled water to obtain 1 mg/mL. After biofilm formation at different times (0, 24 and 48 h), extracts were added, and the plates were further incubated for 24 h at 37 °C. Following incubation, the plates were washed thrice to remove unattached or dead cells. INT (100 μl) was added to all the wells of the plates which were covered and incubated for 30 min in the dark. Afterwards, the absorbance was measured at 490 nm. Percentage inhibition or reduction of the biofilm metabolic activity by the samples was calculated (section 2.5.2).

2.6. Anti-quorum sensing assay

2.6.1. Inhibition of violacein production

The anti-quorum sensing activity of the extracts was performed using 48-well microplates following the protocol described by Blosser and Gray (2000) and Ahmad et al. (2015) with slight modifications. The bacterial suspension was prepared by inoculating a single colony of *Chromobacterium violaceum* ATCC 12472 from an agar plate into 10 mL LB which was incubated in an orbital shaker (140 X g) at 30 °C for 24 h prior to each experiment. Inhibition of violacein production was attained by pipetting 1 mL overnight cultured *C. violaceum* into a 200 mL sterile flask and diluting with 100 mL of Luria-Bertani (LB) standardized (correlating to approximately 1.5×10^8 CFU/mL) by measuring the absorbance of the diluted culture at 590 nm compared to McFarland No. 1 standard. After this, 0.5 mL of LB media was transferred into all wells of a 48-well plate after which 0.5 mg of extracts and positive controls (gentamicin, ciprofloxacin, and amphotericin B) were added to their respective wells to obtain final concentrations ranging from 1.25 to 0.04 mg/mL and 0.25 to 0.008 mg/mL respectively, except for wells designated as blanks (culture and media). Then 0.5 mL of standard overnight culture was added to wells, the plates were sealed with parafilm and incubated in an orbital shaker (140 X g) at 30 °C for 24 h. The MIC values were interpreted as the lowest concentration of samples with no growth inhibition and no pigmentation (purple), while the minimum quorum sensing inhibitory concentration (MQSIC) was measured as the presence of growth (turbid) and no purple pigmentation.

2.6.2. Quantification of violacein

The quorum quenching activity of the extracts was determined using the protocol of Blosser and Gray (2000). The production of violacein by *C. violaceum* ATCC 12472 was measured to evaluate the anti-quorum sensing potential of the extracts. After measuring the inhibition of violacein, the plates were sealed and centrifuged at 4000 x g for 20 min to pellet the bacteria containing violacein (to separate the bacteria). The supernatant was discarded, and the bacterial pellet was resuspended in 1 mL 100 % dimethyl sulfoxide (DMSO). Supernatant (200 μl) was transferred into wells of a 96-well microplate and absorbance was measured at 595 nm. The percentage violacein inhibition was calculated using the following formula:

$$\% \text{ Violacein inhibition} = \left[\left(OD_{\text{control}} - OD_{\text{test}}\right)/OD_{\text{control}}\right] \times 100$$

2.7. Anti-inflammatory activity

2.7.1. 15-Lipoxygenase inhibitory assay

The anti-inflammatory activity was investigated by assessing the lipoxygenase inhibitory activity of extracts (Adebayo et al., 2015). The Tris−HCl buffer was prepared by dissolving 6.57 g of Tris powder into 800 mL of distilled water, and HCl was gradually added to the mixture to obtain a pH of 7.4 and was used in the various reconstitutions of the extracts and positive control. The substrate was prepared by mixing 43.2 μL of linoleic acid + 129.6 μL of 100 % ethanol + 600 μL of Tween-80 + 200 mL of buffer together. One hundred μL of aliquoted 1 mg/mL 15-lipoxygenase (15-LOX) enzyme was added to 900 μL buffer (stock). Xylenol orange (18.75 mg) was mixed with 90 mL of methanol and 10 mL 300 mM sulphuric acid, while 3.79 mg of ferrous sulphate was mixed with 90 mL of methanol + 10 mL 300 mM sulphuric acid. A 200 unit/mL solution of 15-LOX was prepared and kept on ice while preparing for the assay. Plant extracts were prepared (10 mg/mL) in DMSO and reconstituted to 2 mg/mL in the buffer. Quercetin, the positive control, was prepared to 10 mg/mL in distilled water and then reconstituted to 1 mg/mL in buffer. The buffer solution (20 μL) was added to all wells after which the extracts were added only to the first well and serially diluted. After this, 40 μL of the enzyme was added to all wells and incubated for 5 min at 37 °C. Then 40 μL of the substrate was added to all wells except for the wells designated as blanks and further incubated for 20 min. After 20 min incubation, 100 μL of freshly prepared FOX mixture was added and incubated for 30 min. Before reading the plates, 40 μL of the substrate was added to the blank wells. Using a microplate reader, absorbance was measured at 560 nm, and the results were calculated using the following formulae:

$$\% \text{ Enzyme activity} = \frac{\text{Absorbance (sample)} - \text{Absorbance (blank)}}{\text{Absorbance (Negative control)} - \text{Absorbance (blank)}} \times 100$$

% Enzyme inhibition=100-enzyme activity

2.7.2. Determination of nitric oxide (NO) production in LPS-induced RAW 264.7 macrophages

RAW 264.7 macrophages purchased from American Type Culture Collection (ATCC TIB- 71, USA) were used in this study and

maintained in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10 % foetal bovine serum (FBS, Gibco, Sigma) and 1 % penicillin (100 units/mL) and streptomycin (100 ug/mL) (Celtic Molecular Diagnostics) at 37 °C in a 5 % CO_2 atmosphere (HERA cell 150, Thermo Electron Corp., USA). The RAW 264.7 cells were seeded at a density of 4×10^4 cells/well into each well of columns 2 to 11 of sterile tissue culture-treated 96 well plates (NEST, Whitehead Scientific), and incubated for 24 h at 37 °C in 5 % CO_2. Then media was aspirated from all the wells and replaced with fresh medium. The cells were treated with lipopolysaccharide (LPS, Sigma) at a concentration of 1 ug/mL in the presence of different concentrations of the extracts (1.6 to 100 ug/mL) and incubated for 24 h.

2.7.2.1. Measurement of nitrite. The nitric oxide assay was performed according to Yoon et al. (2009). The amount of nitrite, a stable metabolite of nitric oxide (NO) was measured using Griess reagent (Sigma) and the concentration of nitrite in the culture media was used as an indicator of NO production. Briefly, 100 uL of cultured media after 24 h incubation (Section 2.6.2) was transferred into a new plate and 100 ul of Griess reagent (Sigma) was added. The mixture was incubated for 15 min in the dark at room temperature. Then absorbance was measured at 540 nm using a microplate reader (Biotek, Synergy HT). The wells in column 1 and 12 containing only media with Griess reagent were used as the blank in every experiment. The amount of nitrite in the media was calculated from a sodium nitrite ($NaNO_2$) standard curve. The experiment was done in duplicate and repeated 2 times. The percentage of NO inhibition was calculated relative to the untreated LPS induced cells.

2.7.2.2. Assessment of cell viability. The 3-(4, 5-dimethylthiazolyl-2)–2.5-diphenyltetrazolium bromide (MTT) colorimetric assay was used to measure effects on macrophage proliferation and cytotoxic level of the plant extracts using the method described by Mosmann (1983) with slight modification (McGaw et al., 2007). The mitochondrial reduction of MTT to formazan was used as an indicator of cell viability. Briefly, after 24 h of incubation with 1 μg/mL LPS in the presence of different concentrations of the extracts and doxorubicin (Pfizer Labs), the medium was removed from all the wells. RAW 264.7 cells were washed with 200 μL pre-warmed phosphate-buffered saline (PBS) and then 100 μL of fresh media was added. MTT was added (30 μL of a stock solution of 5 mg/mL in PBS) to all the wells and the cells were incubated for 4 h at 37°C in 5 % CO_2. After this, the medium was aspirated from all the wells and DMSO was added to solubilize the formazan salt precipitate and absorbance was measured at a wavelength of 570 nm and a reference wavelength of 630 nm using a microplate reader. The percentage viability of cells treated with extracts and positive control was calculated relative to that of the untreated cells (Mosmann, 1983).

2.8. Antioxidant activity

2.8.1. The 2, 2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay

Antioxidant activity was carried out through radical scavenging activity of the test substances estimated using the quantitative DPPH assay described by Ahmed et al. (2014). Sterile distilled water (40 μL) was measured into 96-well microplates, and 40 μL plant extracts dissolved in methanol was added to the first column and serially diluted. Trolox and vitamin C (ascorbic acid) were included as positive controls. To each well of the microplates, 100 μL of DPPH was added and absorbance was monitored at 517 nm at different time intervals. The percentage scavenging activity was calculated using the formula below:

$$\% \text{ DPPH scavenging activity} = [(A0 - As)/A0] \times 100$$

2.8.2. The 2, 2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) electron reduction assay

The free radical discolouration assay using ABTS exposed to the extracts was performed in 96-well microtitre plates (Ahmed et al., 2014). To each well of the microplates, 40 μL each of sterile distilled water and the plant extracts were added and serially diluted. Trolox and vitamin C (ascorbic acid) were again included as positive controls. One hundred μL of ABTS solution was then added. The mixture was allowed to react for 6 min and absorbance was read at 734 nm. The percentage scavenging activity was calculated using the formula above:

$$\% \text{ DPPH scavenging activity} = [(A0 - As)/A0] \times 100$$

2.9. Cytotoxicity

The MTT assay was used to measure cytotoxicity of the extracts as described in section 7.2.2 using Vero African green monkey kidney cells (Mosmann, 1983). The amount of MTT reduction was measured immediately by reading the absorbance using a microplate reader at a wavelength of 570 nm with a reference wavelength of 630 nm. The wells in column 1, which earlier contained medium and MTT but without cells were used to blank the plate reader. The LC_{50} values were calculated using linear regression as the concentration of test compound resulting in a 50 % reduction of absorbance compared to untreated cells.

2.10. Statistical analysis

The IC_{50} (concentration at which 50 % inhibition occurred) and LC_{50} (50 % lethal concentration) values were determined using linear and non-linear regression curves. Statistically significant differences between experimental groups and controls were evaluated by two-way analysis of variance (ANOVA) with Dunnett's test for multiple comparisons of the means of the extracts to those of the controls. Where $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$, values were considered statistically significant.

3. Results and discussion

3.1. Antimicrobial effects of extracts of Combretum molle

Two of the *Staphylococcus aureus* isolates (SA3 and SA4) were susceptible to all the antibiotics tested (Table 1). SA5 was resistant to four of the ten antibiotics, while SA6 was only resistant to penicillin and was mildly susceptible to three other drugs. The SAA was resistant to four antibiotics, showed mild sensitivity to two and was sensitive to four antibiotics. The SEA was only resistant to penicillin. Ogbuadike et al. (2023) reported that the isolates SA3 and SA4 were resistant to ampicillin while SA4 showed mild sensitivity against amikacin. The reference antibiotics used in this study were gentamicin and ciprofloxacin and the test strains were sensitive to these antibiotics.

The yield of the *Combretum molle* extracts prepared using solvents of different polarities differed (Table 2), with acetone and methanol extracting the highest quantities. According to Mogana et al. (2020), a strong antimicrobial possesses a MIC of ≤0.50 mg/mL. The antibacterial activity of the extracts in this study was moderate to good, with MIC values ranging from 0.02 to 0.63 mg/mL (Table 2). The acetone extract of *C. molle* had the best broad-spectrum activity against all tested organisms (MIC = 0.08 - 0.16 mg/mL). The aqueous cold extract had the best inhibitory activity against SA4 (MIC = 0.02 mg/mL) when compared to gentamicin (MIC = 0.13 mg/mL).

The antibacterial activity of *C. molle* has been reported previously using agar or disk diffusion methods. However, these tests are qualitative and may not give accurate results of the antimicrobial effects

Table 1
Antibiogram result of ten different antibiotics against the test organisms.

Bacterial isolates	Antibiotics tested									
	EFT	PEN	AMP	AMC	OB	OT	CFX/K	KF	RAX	CXM
SA1	S	R	S	S	S	S	S	S	S	S
SA2	S	S	S	R	S	M	S	S	M	S
SA3	S	S	S	S	S	S	S	S	S	S
SA4	S	S	S	S	S	S	S	S	S	S
SA5	R	R	S	S	S	S	R	S	R	S
SA6	S	R	S	S	S	M	M	S	M	S
SAA	M	R	S	S	R	S	R	R	S	M
SEA	S	R	S	S	S	S	S	S	S	S

EFT = ceftiofur; PEN = penicillin G; AMP = ampicillin; AMC = amoxicillin and clavulanic acid; OB = cloxacillin; OT = oxytetracycline; CFX/*K* = (cephalexin and kanamycin); KF = cephalothin; RAX = rifampin; CXM = cefuroxime; *S* = sensitive; *M* = mild sensitivity; *R* = resistant; SA = *S. aureus* isolates from cases of bovine mastitis (SA1–6), SAA = *S. aureus* ATCC 29213, SEA = *S. epidermidis* ATCC 35984.

Table 2
Minimum inhibitory concentration values (mg/mL) of *Combretum molle* extracts.

Extracts	% yield	Bacterial strains and isolates							
		SA1	SA2	SA3	SA4	SA5	SA6	SAA	SEA
MeOH	31.62	0.52±0.18	0.26±0.09	0.31±0.00	0.21±0.09	0.31±0.00	0.16±0.00	0.31±0.00	0.02±0.00
Acetone	35.5	0.08±0.00	0.16±0.00	0.16±0.00	0.16±0.00	0.16±0.00	0.16±0.00	0.08±0.00	0.16±0.00
Ethanol	27.5	0.16±0.13	0.21±0.09	0.31±0.00	0.11±0.05	0.11±0.05	0.16±0.00	0.08±0.00	0.16±0.00
DCM:MeOH	24.9	0.11±0.05	0.16±0.00	0.31±0.00	0.16±0.13	0.16±0.13	0.16±0.00	0.16±0.13	0.07±0.08
AQC	22.7	0.63±0.00	0.63±0.00	0.42±0.18	0.02±0.00	0.47±0.18	0.31±0.00	0.21±0.17	0.11±0.08
AQH	21.8	0.13±0.05	0.31±0.00	0.31±0.00	0.12±0.17	0.26±0.09	0.31±0.00	0.11±0.08	0.16±0.00
Gentamicin	–	0.02±0.00	0.004±0.00	0.004±0.00	0.13±0.00	0.03±0.00	0.004±0.00	0.004±0.00	0.004±0.00
Ciprofloxacin	–	0.004±0.00	0.004±0.00	0.004±0.00	0.004±0.00	0.004±0.00	0.004±0.00	0.004±0.00	0.004±0.00

Dichloromethane: methanol (DCM:MeOH) Aqueous cold and hot extracts (AQC and AQH), *S. aureus* isolates from cases of bovine mastitis (SA1–6), *S. aureus* ATCC 29213 (SAA), *S. epidermidis* ATCC 35984 (SEA), not applicable (-).

of plant extracts with high molecular weight compounds (Bubonja-Sonjie et al., 2020). In 2011, Saidu and Abdullahi reported on the antibacterial efficacy of the aqueous, ethanol and methanol extracts of *C. molle* against *Staphylococcus aureus* strain 0E5 and other bacterial strains using the agar diffusion method, with MIC values ranging between 0.31 and 2.5 mg/mL. Amare and Tadesse (2016) reported the effect of four leaf and seed extracts of *C. molle* against organisms isolated from bovine mastitis using the agar disk diffusion method, with MIC results ranging from 4 mg/disc to 0.25 mg/disk. Kinde et al. (2015) also investigated the stem bark extract of *C. molle* against some clinical isolates of *S. aureus* from bovine mastitis, and the extract at different concentrations showed antibacterial effects against the test isolates with the average zone of inhibition between 12 and 19.5 mm at 10 %, 5 %, 2.5 %, 1.25 % and 0.625 % concentrations. The current study supports prior reports of the antibacterial efficacy of *C. molle* leaf extracts, highlighting their promising MIC values against drug-resistant *S. aureus* isolates from clinical cases of bovine mastitis.

3.2. *The antibiofilm potential of* Combretum molle *extracts via inhibition of biofilm biomass (anti-adhesion)*

Staphylococcus aureus is a well-known biofilm producer and is commonly associated with chronic bovine mastitis infections. The crystal violet assay measures the total biofilm biomass, comprising extracellular polymeric substances produced during biofilm formation that connect or bind to the bacterial cells. The anti-biofilm activity of various substances against *Staphylococcus* spp. isolated from bovine mastitis has been widely investigated. Felipe et al. (2019) investigated the effect of chitosan against isolates associated with chronic mastitis, and the growth of the bacteria was limited by 50.58 %. Similarly, Gonzalez-Ortiz (2014) reported on the effect of wheat bran against *S. aureus* causing mastitis. In their report, the plant inhibited the formation of biofilm at 0.5 % (5 mg/mL). Available literature revealed no previous reports on the antibiofilm (ABF) potential of *C. molle* against mastitis-causing organisms.

The effects of the six different extracts on the development and adhesion of biofilms of the tested mastitis-causing organisms are presented in Table 3. All extracts with greater than 50 % inhibition were considered to have a strong ABF activity. All extracts showed strong inhibition of biofilm formation against SA1 at all test times except AQc at T48. All extracts except AQh showed good inhibitory activity against SA2 at T0 with inhibition >50 %. The AQc extract alone inhibited ≥50 % biofilm biomass formation against SA3 at T0, T24 and T48, also against SA4 at T0 and T24 with inhibition ≥50 % respectively. Biofilm biomass production in SA5 was inhibited with ≥50 % at T0 alone by all extracts except the AQh. All extracts had good to moderate biofilm formation inhibition against SA6 with the AQh extract alone inhibiting biomass formation with inhibition ≥50 % at T0 and T24 respectively. The acetone, ethanol, and AQh extracts, alone inhibited the formation of biofilm biomass with ≥50 % against SEA at T24, and T48 respectively. The acetone, ethanol and AQc extracts had the best biofilm formation inhibition against SAA. In general, the extracts were more easily able to prevent biofilm formation (with better activity at T0) than to disrupt mature biofilm (T48).

3.3. *The antibiofilm potential of* Combretum molle *extracts assessed via* p-*iodonitrotetrazolium violet (INT) metabolic activity*

The viability of the bacterial cells after incubation with test extracts on preformed biofilm as well as on the planktonic cells was ascertained using the INT assay. This was used to interpret the bactericidal effect of the plant extracts on the biofilm metabolic activity. The different extracts of *C. molle* were tested on planktonic cells as well as on preformed biofilm cells to determine their effects on the respiratory activity and the bacterial cell viability of the test bacterial

Table 3
Antibiofilm activity of *Combretum molle* extracts at different times (T0, T24 and T48).

Samples	Hour	% Inhibition							
		SA1	SA2	SA3	SA4	SA5	SA6	SAA	SEA
MeOH	T0	***	***	**	*	***	***	***	**
	T24	***	**	***	**	*	**	*	**
	T48	***	*	***	*	*	**	**	***
Acetone	T0	***	***	*	*	***	***	***	**
	T24	***	*	***	**	*	**	**	***
	T48	***	*	***	*	*	**	***	***
Ethanol	T0	***	***	*	*	***	**	***	**
	T24	***	*	***	**	*	**	**	***
	T48	***	*	***	***	*	**	***	***
DCM:MeOH	T0	***	***	***	***	***	***	***	***
	T24	***	*	**	**	*	**	**	**
	T48	***	*	**	*	*	**	**	**
AQc	T0	***	***	***	***	***	***	***	*
	T24	***	**	***	***	*	***	***	***
	T48	*	*	***	*	*	**	**	**
AQh	T0	**	*	*	*	*	***	*	*
	T24	***	**	***	**	*	**	*	***
	T48	***	*	**	*	*	**	**	***
Gentamicin	T0	***	***	***	***	***	***	***	***
	T24	***	**	***	***	**	***	*	***
	T48	***	**	***	*	*	***	*	*
Ciprofloxacin	T0	***	*	***	*	***	***	*	***
	T24	***	***	***	***	***	***	*	***
	T48	*	***	***	***	*	***	*	***
Amphotericin B	T0	***	***	***	***	***	*	*	***
	T24	***	***	***	***	***	***	*	***
	T48	**	*	***	*	*	**	*	**

***: strong biofilm inhibitors (≥50 % inhibition). **: moderate to poor antibiofilm activity (< 50 % inhibition), *: no activity (0 % inhibition), AQh: hot aqueous extract, AQc: cold aqueous extract.

Table 4
Metabolic activity of the different extracts of *Combretum molle*.

Samples	Hour	Bacterial strains and isolates							
		SA1	SA2	SA3	SA4	SA5	SA6	SAA	SEA
Methanol	T0	***	***	***	***	***	***	***	***
	T24	-*	***	***	***	*	*	***	**
	T48	-*	***	*	***	*	*	*	***
Acetone	T0	***	***	***	***	***	***	***	***
	T24	-*	*	***	**	*	*	*	***
	T48	-*	*	*	*	*	*	*	***
Ethanol	T0	***	***	***	***	***	***	***	***
	T24	-*	***	***	**	*	*	***	***
	T48	-*	**	*	**	*	*	*	***
DCM:MeOH	T0	***	***	***	***	***	***	***	***
	T24	-*	**	***	*	*	*	*	***
	T48	-*	***	*	***	*	*	*	***
AQC	T0	***	***	**	***	***	***	**	***
	T24	-*	*	***	*	**	*	***	***
	T48	-*	***	*	_	*	*	*	***
AQH	T0	**	***	***	***	***	***	***	***
	T24	-*	**	***	***	**	***	***	**
	T48	-*	**	*	***	*	*	***	**
Gentamicin	T0	-*	***	***	***	***	***	***	***
	T24	-*	***	***	***	***	***	***	***
	T48	-*	***	*	***	**	**	*	***
Ciprofloxacin	T0	***	*	***	**	***	***	**	*
	T24	***	*	***	**	**	***	***	***
	T48	***	**	*	***	*	***	*	**
Amphotericin B	T0	-*	***	***	***	***	***	***	***
	T24	-*	***	***	**	**	***	***	***
	T48	***	*	*	***	**	*	*	***

***: strong biofilm inhibitors (≥ 50 % inhibition). **: moderate to poor antibiofilm activity (< 50 % inhibition), * no activity (0 % inhibition), AQh: hot aqueous extract, AQc: cold aqueous extract, DCM: MeOH: dichloromethane: methanol, SA1- 6: *S. aureus* clinical isolates, SAA: *S. aureus* ATCC 29213, SEA: *S. epidermidis* ATCC 35984.

strains after treatment with different extracts and at different times. The results of the metabolic activity assay are shown in Table 4. The extracts and positive controls were tested at a starting concentration of 1 mg/mL and 0.25 mg/mL respectively. At T0 all extracts disrupted the metabolic activity of SA1 by causing a reduction in the cell viability with ≥50 % inhibition. The methanol extract had the best effect on metabolic activity against SA2 at T0, T24 and T48 with ≥50 % inhibition of the viable cells. All extracts followed a similar trend of inhibition of the metabolic activity in SA3 at T0, T24, and T48. The AQh and methanol extracts inhibited the metabolic activity of SA4 at all tested times. The AQc and AQh extracts had comparable results against the inhibition of the bacterial cells of SA5. The SA6 was only inhibited by the AQh extract at both T0 and T24 with an inhibition rate ≥50 %. The acetone, ethanol, DCM: MeOH and the AQc extracts could inhibit the metabolic activity of SEA at T0, T24 and T48. Only the AQh extracts inhibited biofilms at all tested times. Most of the extracts had similar or better inhibition of biofilm metabolic activity with the positive controls. Biofilm metabolic activity of mastitis-causing bacteria enhances the multiplication and spread of the infection and may result in a reoccurrence of the infection which encourages antibiotic resistance.

3.4. *Anti-quorum sensing (QS) potential of the* Combretum molle *extracts*

Bacterial communication is very important in biofilm formation as it is a mechanism to regulate microorganisms that control different virulence factors (González-Ortiz et al., 2014; Al-Haidari et al., 2016). Hence, the different extracts of *C. molle* were investigated against *C. violaceum* in comparison to three antibiotics (Table 5). Amphotericin B, an antifungal agent reported by the Drugbank online (2005) to have no effect on bacterial cells, showed good violacein inhibition and death of the studied bacterial strain in this study. *C. violaceum* can regulate bacterial genes, causing an increase in cell population density, as well as regulating the metabolic activity of the free-living cells which in turn can cause microbial biofilm formation and increase the expression of virulence factors (Abdelhakim et al., 2017). Earlier studies revealed that some medicinal plants could interfere with virulence genes leading to quorum sensing (Al-Haidari et al., 2016; Adeyemo et al., 2022). Although there are no previous reports on the anti-quorum sensing activity of *C. molle*, some species in the Combretaceae family have been reported to possess quorum quenching ability (Asfour 2018; Vandeputte et al., 2010).

The extracts of *C. molle* had good to moderate violacein inhibition at different concentrations (Table 5). The methanol, acetone, and ethanol extracts had the best violacein inhibition at all tested concentrations (2.5 - 0.08 mg/mL) with inhibition values ranging between 69.37 and 95.07 %. The different extracts not only inhibited the production of violacein, but also had a bactericidal effect on the strain, at 0.63 and 0.31 mg/mL respectively. The quorum quenching effect of the extracts, although not as significant as that of the positive controls, was promising.

3.5. *Lipoxygenase inhibition and antioxidant potential of* Combretum molle *extracts*

The hot water extract of *C. molle* had the best lipoxygenase inhibitory activity (85.26 %) which was higher than that of the positive control (quercetin 82.88 %) (Table 6). Antioxidants can destroy reactive oxygen species and disrupt the free radical chain of reaction before they cause damage to the cells (Hakiman and Maziah 2009; Gupta, 2015). The DPPH and ABTS non-enzymatic antioxidant assays were conducted. The antioxidant potential of the different extracts of *C. molle* expressed as IC_{50} against the DPPH and ABTS radicals is presented in Table 6. The different extracts had good to moderate antioxidant activity against the DPPH and ABTS radicals with IC_{50} values

Table 5
Percentage inhibition of violacein production in *Chromobacterium violaceum* by *Combretum molle* extracts.

Extracts	Concentrations (mg/mL)						MIC (mg/mL)	MQSIC (mg/mL)
	0.08	0.16	0.31	0.63	1.25	2.5		
MeOH	77.26±0.01	82.49±0.01	83.69±0.02	94.53±0.00	94.92±0.00	95.28±0.00	0.31	0.08
Acetone	76.04±0.03	81.82±0.02	87.37±0.04	92.51±0.01	92.48±0.06	92.87±0.00	0.63	0.08
Ethanol	69.37±0.04	65.79±0.02	73.86±0.02	92.17±0.00	93.28±0.00	95.07±0.01	0.31	0.08
DCM:MeOH	45.98±0.13	61.02±0.11	72.87±0.01	93.05±0.00	93.28±0.00	94.89±0.00	0.31	0.08
AQc	45.33±0.21	70.38±0.03	80.13±0.01	94.99±0.00	95.95±0.00	96.12±0.00	0.31	0.16
AQh	32.81±0.03	32.47±0.18	62.47±0.01	78.94±0.03	95.77±0.00	95.90±0.00	0.63	0.16
Ciprofloxacin	95.31	96.08	96.16	96.24	96.24	96.32	0.03	0.03
Amphotericin B	94.99	96.06	96.16	96.24	96.29	96.29	0.03	0.03
Gentamicin	75.03	90.82	94.27	95.18	96.29	96.27	0.25	0.03

MQSIC= Minimum quorum sensing inhibitory concentration, MIC= Minimum inhibitory concentration, AQh: hot aqueous extract, AQc: cold aqueous extract.

Table 6
Anti-lipoxygenase (% inhibition) and antioxidant activity (IC_{50} in μg/mL) of extracts of *C. molle*.

Extracts	LOX	DPPH	ABTS
MeOH	64.08±0.001****	7.79±2.48	6.78±2.27
Acetone	57.85±0.02****	10.60±1.45**	5.48±2.86
Ethanol	63.42±0.004****	8.19±3.22	4.16±2.90
DCM:MeOH	61.18±0.001****	10.35±4.62**	3.94±1.64
AQc	67.30±0.01****	15.39±3.45****	2.34±0.38
AQh	87.26±0.02****	21.51±3.12****	5.89±1.85
Quercetin	82.88±0.05	–	–
Trolox	–	3.47±2.26	3.02±2.26
Vitamin C	–	2.83±0.20	1.20±0.20

- = not tested, AQh: hot aqueous extract, AQc: cold aqueous extract. Statistical analysis was performed with Dunnett's multiple comparisons test using two-way ANOVA; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$.

ranging from 7.79 - 21.51 and 2.34–6.78 μg/mL respectively. The cold water extract showed good scavenging ability (2.34 μg/mL) of the ABTS radical when compared to that of the positive control (Trolox, 3.02 μg/mL).

3.6. *Inhibition of nitric oxide (NO) production by C.* molle

The production of nitric oxide is implicated in or prolongs inflammatory diseases (Wayne and Coleman, 2001). The% inhibition of NO and the viability of the cells following exposure to *C. molle* extracts are presented in Table 7. The extracts showed inhibition or reduction of NO production against the LPS-induced RAW 264.7 macrophage cells at different concentrations. The cold-water extract at 100 μg/ml and 50 μg/mL, had the best NO inhibition (88.17 % and 86 %) with 80.05 % and 80.89 % cell viability respectively. At 12.5 μg/ml, the methanol extract had the highest NO inhibition (75.26 %) and cell viability of 89.46 %. Also, the MeOH, acetone and cold water extracts had better NO inhibition at this concentration when compared to the control (quercetin). At the lowest concentration (1.6 μg/mL), only the cold water extract could inhibit NO production by 18.57 %, with cell viability of 86.82 %.

3.7. *Cytotoxicity of* Combretum molle *extracts*

An extract is considered non-cytotoxic if the LC_{50} is $\geq$ 0.02 mg/mL (Zirihi et al., 2005). The metabolic activity or the change in cell population or change in mass of a cell can be used to measure cytotoxicity (McGaw et al., 2014). The acetone, ethanol, and DCM: MeOH extracts were least toxic to Vero cells ($LC_{50} \geq 1$) (Table 8). The selectivity index (SI), defined as the ratio of toxicity (LC_{50}) to antibacterial activity (MIC), helps to differentiate activity of the plant in relation to potential toxicity (Adamu et al., 2014). Extracts with SI greater than one are more active to the target organism than they are toxic to cells, which is promising for further development of safe plant-based therapeutic preparations (Makhafola et al., 2012). All extracts had good to moderate SI values (Table 8) ranging 1.59 to as high as 50, with the acetone extract having the best SI values against most of the test organisms and thus the most promising potential for further development as a treatment against *S. aureus* infections.

4. Conclusion

Antimicrobial resistance is a growing threat to human and animal welfare, and alternative or complementary measures are needed to reduce the impact of this phenomenon. Mastitis, caused by a variety of pathogens including *Staphylococcus aureus,* is a significant economic problem in the dairy industry, resulting in adverse effects on animal welfare and food security. In this study, *S. aureus* strains isolated from clinical cases of bovine mastitis had varying degrees of

Table 7
Percentage inhibition of nitric oxide production (and cell viability in brackets) by *Combretum molle* extracts in RAW 264.7 macrophage cells.

Extracts	Concentration (μg/mL)			
	100	50	12.5	1.6
MeOH	79.65±0.00 (73.58±1.90)	77.25±0.00 (83.20±2.4)	75.26±0.00 (89.46±2.4)	0.00 (92.49±2.4)
Acetone	80.45±0.00 (66.03±1.7)	77.47±0.00 (79.28±1.9)	68.22±0.00 (87.25±2.3)	0.00 (98.93±2.7)
Ethanol	75.22±0.00 (68.05±1.7)	35.27±0.00 (74.99±2.1)	0.00± (88.87±2.5)	0.00±0.00 (101.85±2.7)
DCM:MeOH	71.70±0.00 (67.15±1.8)	71.78±0.00 (74.89±1.9)	16.26±0.7 (91.55±2.5)	0.00 (98.37±2.5)
AQc	88.17±0.00 (80.05±2.0)	86.00±0.00 (80.89±2.2)	71.92±0.00 (93.28±2.4)	18.57±0.00 (86.82±2.3)
AQh	85.11±0.00 (63.03±1.6)	81.93±0.00 (72.62±1.8)	61.20±0.00 (89.60±2.2)	0.00 (98.25±2.6)
Quercetin/(MTT)	96.15±0.8 (31.52±3.3)	94.53±4.9 (54.99±4.6)	64.66±5.6 (81.1 ± 4.4)	43.91±3.5 (99.00±9.3)

AQH: hot aqueous extract, AQC: cold aqueous extract.

Table 8
LC_{50} values against Vero cells and selectivity index (SI = LC_{50}/MIC) of the extracts of *Combretum molle*.

Extracts	LC_{50} (mg/mL)	Bacterial strains and isolates (SI)							
		SA1	SA2	SA3	SA4	SA5	SA6	SAA	SEA
MeOH	1	1.92	3.85	3.23	4.76	3.23	6.25	3.23	50
Acetone	1	12.5	6.25	6.25	6.25	6.25	6.25	12.5	6.25
Ethanol	1	6.25	4.76	3.23	9.09	9.09	6.25	12.5	6.25
DCM:MeOH	1	9.09	6.25	3.23	6.25	6.25	6.25	6.25	14.29
AQH	1	7.69	3.23	3.23	8.33	3.85	3.23	9.09	6.25
AQC	1	1.59	1.59	2.38	50	2.13	3.23	4.76	9.09
Doxorubicin	0.01±0.00	–	–	–	–	–	–	–	–

- = not tested, AQH: hot aqueous extract, AQC: cold aqueous extract.

resistance to a panel of antibiotics, with nearly all of them being resistant to penicillin. Leaf extracts of *Combretum molle* had promising antibacterial activity against these isolates as well as standard ATCC strains, while lacking noteworthy toxicity to mammalian cells. The ability of *C. molle* extracts to inhibit both quorum sensing and biofilm formation provides further evidence of the potential of *C. molle* to reduce development of bacterial resistance. Water extracts of *C. molle* generally had good in vitro antioxidant and anti-inflammatory activity, which are additional useful properties, as oxidative stress and inflammation are commonly associated with bacterial infections. The bioactivity of the extracts also supports the use of the plant in traditional medicine against infections and inflammation. Further work aims to explore *C. molle* extracts and active fractions in combination with other bioactive plant extracts towards the formulation of affordable, non-toxic herbal products for the management of bovine mastitis. Phytochemical investigations to unravel the chemical complexity and identify possible active compounds of *C. molle* leaf extracts are also underway.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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References

Abdelhakim, B., Nadia, D., Abdeslam, Et-T., Jamal, A., Youssef, B., 2017. Medicinal plant products targeting quorum sensing for combating bacterial infections. Asian Pac. J. Trop. Med. S1995-7645, 30205-5.
Abera, A., 2020. Prevalence and associated risk factors of bovine mastitis in lactating cows of small holder dairy farms in Ethiopia. J. Veterin. Med. Surg. 4, 2–27.
Adamu, M., Naidoo, V., Eloff, J.N., 2014. The antibacterial activity, antioxidant activity and selectivity index of leaf extracts of thirteen South African tree species used in ethnoveterinary medicine to treat helminth infections. BMC Vet. Res. 10, 52.
Adebayo, S.A., Dzoyem, J.P., Shai, L.J., Eloff, J.N., 2015. The anti-inflammatory and antioxidant activity of 25 plant species used traditionally to treat pain in southern Africa. BMC Complement. Altern. Med. 15, 159.
Adeniran, L.A., Okpi, S., Anjorin, T.S., Ajagbonna, O.P., 2020. Medicinal plants used in ethnoveterinary practices in the Federal Capital Territory, North-Central Nigeria. J. Med. Plant. Res. 14, 377–388.
Adeyemo, R.O., Famuyide, I.M., Dzoyem, J.-P., McGaw, L.J., 2022. Anti-biofilm, antibacterial, and anti-quorum sensing activities of selected South African plants traditionally used to treat diarrhoea. Evid.-Based Complement. Altern. Med. 1307801, 12.
Ahmad, A., Viljoen, A., Chenia, H., 2015. The impact of plant volatiles on bacterial quorum sensing. Lett. Appl. Microbiol. 60, 8–19.
Ahmed, A.S., McGaw, L.J., Moodley, N., Vinasan Naidoo, V., Eloff, J.N., 2014. Cytotoxic, antimicrobial, antioxidant, anti-lipoxygenase activities and phenolic composition of *Ozoroa* and *Searsia* species (Anacardiaceae) used in South African traditional medicine for treating diarrhoea. S. Afr. J. Bot. 95, 9–18.
Alberta Milk, 2019. What Causes Mastitis in Dairy Cows?. https://albertamilk.com/ask-dairy-farmer/what-causes-mastitis-in-dairy-cows/. Accessed on 26/4/2019.
Al-Haidari, R.A., Shaaban, M.I., Ibrahim, S.R.M., Mohamed, G.A., 2016. Anti-quorum sensing activity of some medicinal plants. Afr. J. Tradition. Complem. Alternat. Med. 13, 67–71.
Alhussien, M.N., Dang, A.K., 2018. Milk somatic cells, factors influencing their release, future prospects, and practical utility in dairy animals: an overview. Vet. World 11 (5), 562–577.
Ali-Vehmas, T., Westphalen, P., Myllys, v., Sandholm, M., 1997. Binding of *Staphylococcus aureus* to milk fat globules increases resistance to penicillin-*G*. J. Dairy Res. 64, 253–260.
Amare, T., Tadesse, E., 2016. In vitro antibacterial effect of *Combretum molle* and Fr1 against *Staphylococcus aureus* and *E. coli* isolated from bovine mastitis. World J. Biol. Med. Sci. 3, 115–131.
Anato, M., Bezabih, M., Ketema, T., 2018a. Effect of *Combretum molle* (Combretaceae) seed extract on hematological and biochemical parameters. J. Med. Plant Res. 12, 55–63.
Anato, M., Ketema, T., 2018b. Anti-plasmodial activities of *Combretum molle* (Combretaceae) [Zwoo] seed extract in Swiss albino mice. BMC Res. Note. 11, 312.
Asfour, H.Z., 2018. Anti-quorum sensing natural compounds. J. Microsc. Ultrastruct. 6, 1–10.
Banga, C.B., Neser, F.W.C., Garrick, D.J., 2014. The economic value of somatic cell count in South African Holstein and Jersey cattle. S. Afr. J. Anim. Sci. 44, 173–177.
Blosser, R.S., Gray, K.M., 2000. Extraction of violacein from *Chromobacterium violaceum* provides a new quantitative bioassay for N-acyl homoserine lactone autoinducers. J. Microbiol. Method. 40, 47–55.
Bubonja-Sonjie, M., Knežević, S., Abram, M., 2020. Challenges to antimicrobial susceptibility testing of plant-derived polyphenolic compounds. Arch. Indust. Hyg. Toxic. 71, 300–311.
Chitura, T., Muvhali, P.T., Shai, K., Mushonga, B., Kandiwa, E., 2018. Use of medicinal plants by livestock farmers in a local municipality in Vhembe District, South Africa. Appl. Ecol. Environ. Res. 16, 6589–6605.
CLSI (Clinical Laboratory Standards Institute), 2020. CLSI Supplement M100. Performance Standards For Antimicrobial Susceptibility Testing, 30th ed. Clinical and Laboratory Standards Institute, USA.
Drugbank, 2005. Amphotericin B. https://go.drugbank.com/drugs/DB00681. Accessed on the 24/05/2023.
Eloff, J.N., 1998a. Which extractant should be used for the screening and isolation of antimicrobial components from plants? J. Ethnopharmacol. 60, 1–8.
Eloff, J.N., 1998b. A sensitive and quick method to determine the minimum inhibitory concentration of plant extracts for bacteria. Planta Med. 64, 711–713.
Erhabor, R.C., Erhabor, J.O., McGaw, L.J., 2019. The potential of South African medicinal plants against microbial biofilm and quorum sensing of foodborne pathogens: a review. S. Afr. J. Bot. 126, 214–231.
Felipe, V., Breser, M.L., Bohl, L.P., Rodrigues da Silva, E., Morgante, C.A., Correa, S.G., Porporatto, C., 2019. Chitosan disrupts biofilm formation and promotes biofilm eradication in *Staphylococcus* species isolated from bovine mastitis. Int. J. Biol. Macromol. 126, 60–67.
Garba, M.H., Sherifat, M.L., Abdul-Majid, A.O., Hafsa, L.M., Bulus, L.J., Yusuf, G., 2018. Therapeutic evaluation of *Combretum molle* (Combretaceae) (bush willow) stem-bark extract in experimental avian coccidiosis. J. Asian Nat. Prod. Res. 1, 1–9.
Gemechu, T., Yunus, H., A., Soma, M., Beyene, A., 2019. Bovine mastitis: prevalence, Isolation and identification of major bacterial pathogens in selected areas of Bench Maji Zone, Southwest Ethiopia. J. Veterin. Med. Anim. Health 11, 2.
González-Ortiz, G., Quarles Van Ufford, H.C., Halkes, S.B.A., Cerdà-Cuéllar, M., Beukelman, C.J., Pieters, R.J., Liskamp, R.M.J., Pérez, J.F., Martín-Orue, S.M., 2014. New properties of wheat bran: anti-biofilm activity and interference with bacteria quorum-sensing systems. Environ. Microbiol. 16, 1346–1353.
Gupta, D., 2015. Methods for determination of antioxidant capacity: a review. Int. J. Pharmacol. Sci. Res. 6, 546–566.
Hakiman, M., Maziah, M., 2009. Non-enzymatic antioxidant activities in aqueous extract of different *Ficus deltoidea* accessions. J. Med. Plant. Res. 3, 120–131.
Kebede, T., Gadisa, E., Tufa, A., 2021. Antimicrobial activities evaluation and phytochemical screening of some selected medicinal plants: a possible alternative in the treatment of multidrug-resistant microbes. PLoS One 16, e0249253.

Karzis, J., Petzer, I.M., Donkin, E.F., Naidoo, V., 2018. Proactive udder health management in South Africa and antimicrobial resistance monitoring of *Staphylococcus aureus* in dairy herds from 2001 to 2010. J. S. Afr. Veterin. Assoc. 89, 1–8.

Khan, R.S.M., Akhter, T., Hussain, M., 2021. Ethno-veterinary practice for the treatment of animal diseases in Neelum Valley, Kashmir Himalaya, Pakistan. PLoS One 16, e0250114.

Khunoana, E.T., Madikizela, B., Erhabor, J., Nkadimeng, S.M., Arnot, L.F., Van Wyk, I., McGaw, L.J., 2019. A survey of plants used to treat livestock diseases in the Mnisi community, Mpumalanga, South Africa, and investigation of their antimicrobial activity. S. Afr. J. Bot. 126, 21–29.

Kinde, H., Regassa, F., Wubie, A., Asaye, M., 2015. The in-vitro antibacterial effect of three selected plant extracts against *Staphylococcus aureus* and *Streptococcus agalactiae* isolated from bovine mastitis. J. Veterin. Sci. Technol. S13.

Klančnik, A., Podobnik, P., Možina, S.S., Raspor, P., Bohinc, K., Jeršek, B., 2014. Determination of viable biofilm cells in microtiter plates. II International Congress, "Food Technology, Quality and Safety" Novi Sad, Serbia. Proceedings., pp. 252–257.

Kobayashi, S.D., Malachowa, N., DeLeo, F.R., 2015. Pathogenesis of *Staphylococcus aureus* abscesses. Am. J. Pathol. 185, 1518–1527.

Makhafola, T.J., Babatunde, B.S., Esameldin, E.E., Eloff, J.N., 2012. Ochnaflavone and ochnaflavone 7-0- methyl ether two antibacterial biflavonoids from *Ochna pretoriensis* (Ochnaceae). Nat. Prod. Commun. 7, 1601–1604.

Maphosa, V., Masika, P.J., 2010. Ethnoveterinary uses of medicinal plants: a survey of plants used in the ethnoveterinary control of gastro-intestinal parasites of goats in the Eastern Cape Province, South Africa. Pharmaceut. Biol. 48, 697–702.

McGaw, L.J., Rabe, T., Sparg, S.G., Jäger, A.K., Eloff, J.N., Van Staden, J., 2001. An investigation on the biological activity of *Combretum* species. J. Ethnopharmacol. 75, 45e50.

McGaw, L.J., Steenkamp, V., Eloff, J.N, 2007. Evaluation of *Athrixia* bush tea for cytotoxicity, antioxidant activity, caffeine content and presence of pyrrolizidine alkaloids. J. Ethnopharmacol. 110, 16–22.

McGaw, L.J., Eloff, J.N., 2008. Ethnoveterinary use of southern African plants and scientific evaluation of their medicinal properties. J. Ethnopharmacol. 119, 559–574.

McGaw, L.J., Famuyide, I.M., Khunoana, E.T., Aremu, A.O., 2020. Ethnoveterinary botanical medicine in South Africa: a review of research from the last decade (2009 to 2019). J. Ethnopharmacol. 257, 112864.

McGaw, L.J., Elgorashi, E.E., Eloff, J.N., 2014. Cytotoxicity of African medicinal plants against normal animal and human cells. In: Kuete, V. (Ed.), Toxicological Survey of African Medicinal Plants. Elsevier, Amsterdam, pp. 181–233.

Mogana, R., Adhikari, A., Tzar, M.N., Ramliza, R., Wiart, C., 2020. Antibacterial activities of the extracts, fractions and isolated compounds from *Canarium patentinervium* Miq. against bacterial clinical isolates. BMC Complem. Med. Therap. 20, 55.

Mogashoa, M.M., Masoko, P., Eloff, J.N., 2019. Different *Combretum molle* (Combretaceae) leaf extracts contain several different antifungal and antibacterial compounds. S. Afr. J. Bot. 126, 322–327.

Mosmann, T., 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. Method. 65, 263–271.

Mussarat, S., Amber, R., Tariq, A., Adnan, M., et al., 2014. Ethnopharmacological assessment of medicinal plants used against livestock infections by the people living around Indus River. Biomed. Res. Int. 2014, 616858.

Ogbuadike, E.C., Nkadimeng, S.M., Igwe, C.C., Dzoyem, J.-P., Qekwana, D.N., Petzer, I.-M., McGaw, L.J., 2023. An in vitro study on the potential of selected South African plant extracts to prevent and treat bovine mastitis. S. Afr. J. Bot. 154, 98–107.

Pedersen, R.R., Krömker, V., Bjarnsholt, T., Dahl-Pedersen, K., Buhl, R., Jørgensen, E., 2021. Biofilm research in bovine mastitis. Front. Veterin. Sci. 8, 656810.

Petrovski, K.R., Trajcev, M., Buneski, G.A., 2006. Review of the factors affecting the costs of bovine mastitis. J. S. Afr. Vet. Assoc. 77, 52–60.

Phophi, L., Petzer, I.M., Qekwana, D.N., 2019. Antimicrobial resistance patterns and biofilm formation of coagulase-negative *Staphylococcus* species isolated from subclinical mastitis cow milk samples submitted to the Onderstepoort Milk Laboratory. BMC Vet. Res. 15, 420.

Rademan, S., Anantharaju, P.G., Madhunapantula, S.V., Lall, N., 2019. The anti-proliferative and antioxidant activity of four indigenous South African plants. Afr. J. Traditi. Complem. Alternat. Med. 16, 13–23.

Rani, P., Khullar, N., 2004. Antimicrobial evaluation of some medicinal plants for their anti-enteric potential against multi-drug resistant *Salmonella typhi*. Phytother. Res. 18, 670–673.

Regassa, F., Araya, M., 2012. In vitro antimicrobial activity of *Combretum molle* (Combretaceae) against *Staphylococcus aureus* and *Streptococcus agalactiae* isolated from crossbred dairy cows with clinical mastitis. Trop. Anim. Health Product. 44, 1169–1173.

Saidu, T.B., Abdullahi, M., 2011. Phytochemical determinations and antibacterial activities of the leaf extracts of *Combretum molle* and *Gossypium arboretum*. Bayero J. Pure Appl. Sci. 4, 132–136.

Sandasi, M., Leonard, C.M., Viljoen, A.M., 2010. The in vitro antibiofilm activity of selected culinary herbs and medicinal plants against *Listeria monocytogenes*. Lett. Appl. Microbiol. 50, 30–35.

Sserunkuma, P., McGaw, L.J., Nsahlai, I.V., Van Staden, J., 2017. Selected southern African medicinal plants with low cytotoxicity and good activity against bovine mastitis pathogens. S. Afr. J. Bot. 111, 242–247.

Ugboko, H.U., Nwinyi, O.C., Oranusi, S.U., Fatoki, T.H., Conrad, A., Omonhinmin, C.A., 2020. Antimicrobial importance of medicinal plants in Nigeria. Sci. World J. 7059323, 10.

Vandeputte, O.M., Kiendrebeogo, M., Rajaonson, S., Diallo, B., Mol, A., El Jaziri, M., Baucher, M., 2010. Identification of catechin as one of the flavonoids from *Combretum albiflorum* bark extract that reduces the production of quorum-sensing-controlled virulence factors in *Pseudomonas aeruginosa* PAO1. Appl. Environ. Microbiol. 76, 243–253.

Vasudevan, P., Nair, M.K., Annamalai, T., Venkitanarayanan, K.S., 2003. Phenotypic and genotypic characterization of bovine mastitis isolates of *Staphylococcus aureus* for biofilm formation. Vet. Microbiol. 92, 179–185.

Viguier, C., Arora, S., Gilmartin, N., Welbeck, K., O'Kennedy, R., 2009. Mastitis detection: current trends and future perspectives. Trend. Biotechnol. 27, 486–493.

Wayne, P.A., Coleman, J.W., 2001. Nitric oxide in immunity and inflammation. Int. Immunopharmacol. 1, 1397–1406.

Yoon, W.-J., Kim, S.-S., OH, T.-H., Lee, N.H., 2009. *Cryptomeria japonica* essential oil inhibits the growth of drug-resistant skin pathogens and LPS-induced nitric oxide and pro-inflammatory cytokine production. Pol. J. Microbiol. 58, 61–68.

Zirihi, G.N., Mambu, L., Guede-Guina, F., Bodo, B., Grellier, P., 2005. In vitro antiplasmodial activity and cytotoxicity of 33 West African plants used for treatment of malaria. J. Ethnopharmacol. 98, 281–285.