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Selected South African *Combretum* spp. extracts inhibit methicillin-resistant *Staphylococcus aureus* and ESBL strains of *Escherichia coli* and *Klebsiella pneumoniae*

Locklen Grimsey[a], Sandy F. Van Vuuren[b], Mitchell Henry Wright[a], Ian Edwin Cock[a,c,*]

[a] School of Environment and Science, Griffith University, 170 Kessels Rd, Nathan, Queensland 4111, Australia
[b] Department of Pharmacy and Pharmacology, Faculty of Health Sciences, University of the Witwatersrand, Parktown, Gauteng 2193, South Africa
[c] Centre for Planetary Health and Food Security, Nathan Campus, Griffith University, 170 Kessels Rd, Nathan, Queensland 4111, Australia

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ABSTRACT

An increase in antibiotic resistance and a corresponding decrease in antimicrobial drug discovery have resulted in researchers focussing on alternative therapies, including plant based medicines. The development of bacterial strains resistant to β-lactam, including resistance to the second generation drugs methicillin, is particularly concerning and has rendered many common therapies ineffective or of substantially decreased efficacy. New antibiotic therapies are urgently needed. The antibacterial activity of selected southern African *Combretum* spp. leaf extracts towards β-lactam resistant and sensitive bacterial strains was investigated by disc diffusion assays and quantified by broth microdilution Minimum inhibitory concentration (MIC) assays. Toxicity was evaluated by testing *Artemia* nauplii mortality and human dermal fibroblast (HDF) cytotoxicity assays. The gas chromatography coupled to Mass spectrometry (GC-MS) headspace analysis was used to identify volatile terpenoid components. The leaf extracts of all *Combretum* spp. displayed noteworthy growth inhibitory activity against all of the bacteria tested, including against Methicillin resistant *Staphylococcus aureus* MRSA, and the extended spectrum β-lactamase (ESBL) strains of *E. coli* and *K. pneumoniae*. *Combretum heroense* leaf extract had particularly good (MICs = 170-680 μg/mL) antibacterial activity against all bacterial strains. Notably, this extract was a better inhibitor of MRSA than of the β-lactam sensitive strain of *S. aureus*. The *Combretum vendae* extract was also a good inhibitor of the MRSA and ESBL strains. Noteworthy antibacterial activity (MICs 120-890 μg/mL) was also noted for *Combretum collinum* and *Combretum molle* extracts against all bacteria, and for the *Combretum bracteosum* and *Combretum erythrophyllum* extracts against the MRSA and ESBL strains (500-1625 μg/mL). All extracts were non-toxic in the *Artemia* nauplii lethality assay (ALA) and HDF assays and the calculated therapeutic indexes (TIs) indicated their safety for use as antibiotic chemotherapies. The GC-MS headspace analysis identified and highlighted several noteworthy monoterpenoids which had antibacterial properties. In particular, cineole, terpineol, camphor, borneol and limonene were present in relative abundance in all *Combretum* spp. extracts. Further phytochemical and mechanistic studies of these extracts are warranted.

1. Introduction

The rapid development of bacterial resistance to multiple antibiotics in recent years has decreased the efficacy of many therapeutics, and in some cases has rendered them completely ineffective against some clinically important pathogens (Cheesman et al., 2017). Bacteria have evolved rapidly to produce β-lactamase enzymes that degrade the β-lactam structure of penicillin and other first-generation β-lactam antibiotics. In response, synthetic chemists adapted and modified the β-lactam structural scaffold by adding a variety of functional groups, resulting in the production of later generation β-lactam antibiotics (e.g. methicillin, oxacillin). Whilst these antibiotics are based on β-lactam scaffolds, the modified structures allowed them to evade the actions of the original bacterial β-lactamase enzymes, which degrade other β-lactam antibiotics. This has allowed these modified β-lactam antibiotics to function with high efficacy, even in bacterial strains that produce β-lactamase enzymes. However, misuse and

Abbreviations: ALA, *Artemia* lethality assay; CAM, complementary and alternative medicine; ESBL, extended spectrum β-lactamase; HDF, human dermal fibroblasts; LC_{50}, the concentration that induces 50% lethality; MIC, minimum inhibitory concentration; MRSA, methicillin resistant *Staphylococcus aureus*; ZOI, zone of inhibition

* Corresponding author at: School of Environment and Science, Griffith University, 170 Kessels Rd, Nathan, Queensland 4111, Australia.
E-mail address: I.Cock@griffith.edu.au (I.E. Cock).

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overuse of the later generation β-lactam antibiotics has provided selective pressure and numerous bacterial pathogens have evolved to produce further β-lactamase enzymes with substantially broader specificity. These enzymes can bind and inactivate most β-lactam antibiotics (including methicillin and oxacillin), thereby rendering them of low efficacy. The development of methicillin-resistant strains of *S. aureus* (MRSA) in particular has resulted in dramatic increases in *S. aureus*-associated mortality. Indeed, MRSA infections now cause more death globally than the human immunodeficiency virus (HIV) or emphysema (Chew et al., 2018). Similarly, multiple bacterial pathogens (particularly in the family Enterobacteriaceae) have now developed high activity extended spectrum β-lactamase (ESBL) enzymes that provide them with resistance to a broad array of β-lactam antibiotics. Chemotherapeutic options for the treatment of many antibiotic-resistant strains are now limited and vancomycin is often the only effective therapeutic option (particularly for MRSA). However, the increased usage of vancomycin has resulted in the development of several vancomycin-resistant MRSA strains (Hasan et al., 2016). New effective and safe antibiotic therapies that inhibit the growth of antibiotic-resistant bacterial strains are required and the World Health Organisation (WHO) considers this to be amongst the most urgent challenges currently facing medical science (World Health Organization, 2022).

Traditional plant-based therapies have been used for thousands of years to treat pathogenic diseases and a re-examination of the species used in those therapies may provide promising leads for the development of new antibiotics. Plants of the family Combretaceae are promising for the discovery of new antibiotic chemotherapies due to their widespread usage to treat a broad range of pathogenic diseases. The Combretaceae consists of 18 genera, of which *Combretum* (~370 species) and *Terminalia* (~200 species) are the largest and most diverse (Cock, 2015; Cock et al., 2022). Species from both genera are widely used therapeutically for a variety of purposes, including for the treatment of numerous pathogenic diseases (Van Wyk et al., 2009). The therapeutic uses of many southern African *Combretum* species (including *Combretum bracteosum* (Hochst.) Engl. & Diels, *Combretum collinum* Fresen., *Combretum erthrophyllum* Gilg. & Ledermann ex Engl., *C., Combretum hereroense* Schinz., *Combretum molle molle* R.Br ex G.Don and *Combretum vendae* A.E.van Wyk) have been particularly well documented and include uses for the treatment to colds, cough, venereal diseases, infertility, diarrhoea, dysentery, sores and wounds (Watt and Breyer-Brandwijk, 1962; Hutchings, 1996; Eloff, 1999; Cock, 2015).

The effects of *Combretum* spp. extracts on numerous bacteria have also been verified experimentally. An early study screened acetone leaf extracts of 22 South African *Combretum* spp. (including *C. molle, Combretum moggii* Exell., *C. erythrophyllum, Combretum padoides* Engl. & Diels, *Combretum paniculatum* Vent. and *Combretum mossambicense* (Klotzch) Engl.) against a panel of bacteria and reported noteworthy inhibitory activity against *Staphylococcus aureus, Escherichia coli, Enterococcus faecalis* and *Pseudomonas aeruginosa* (Eloff, 1999). Another study reported strong growth inhibitory activity for *Combretum fragrans* F. Hoffm., *C. hereroense, C. molle, C. padoides* and *Combretum zeyheri* Sond. extracts against *S. aureus, Eneterbacter aerogenes, Staphylococcus epidermidis, Bacillus subtilis* and *Micrococcus luteus* (Fyhrquist et al., 2002). More recently, *C. collinum, C. erythrophyllum, C. hereroense, C. microphyllium* and *C. molle* leaf extracts were reported to strongly inhibit the growth of sixteen bacterial pathogens, including *Alcaligenes faecalis, Aeromonas hydrophila, Bacillus cereus, B. subtilis, Pseudomonas fluorescens, Proteus vulgaris, Shigella sonnei* and *S. aureus* (Cock and Van Vuuren, 2015). Notably, whilst these studies reported noteworthy antibacterial activity against a broad-spectrum of bacterial pathogens, all focussed on the effects of *Combretum* spp. extracts against bacteria susceptible to most clinically used antibiotics. Studies examining the effects of *Combretum* spp. extracts against methicillin-resistant and extended spectrum β-lactamase (ESBL) producing bacteria have been neglected. With the rapid development of multiple-antibiotic resistant strains of bacteria in recent years, it is increasingly important to develop new antibiotic therapies that are effective against bacteria that are refractory to the effects of commonly used clinical therapies. This study aimed to address this gap in the literature by testing the growth inhibitory effects of selected *Combretum* spp. extracts against methicillin-resistant *S. aureus* and some ESBL bacteria. Specifically, due to the antibacterial activity of southern African *Combretum* spp. against multiple bacterial pathogens, it is likely that some species will also inhibit the growth of antibiotic-resistant strains of the same bacteria. Our study aimed to investigate the antibiotic potential of extracts prepared from selected southern African *Combretum* spp. against some β-lactam sensitive bacteria, and compare the results to their activity against antibiotic-sensitive strains.

2. Methods

2.1. Sourcing of plant samples and preparation of extracts

Selected *Combretum* spp. were sourced from trees in South African botanical gardens identified by resident botanists (Table 1). *Combretum bracteosum* (Hochst.) Engl. & Diels leaves were collected from the Walter Sisulu National Botanical Gardens, Johannesburg under the guidance of the Associate Curator, Andrew Hankey. All other *Combretum* spp. leaves were harvested at the Lowveldt National Botanical Gardens, Mbombela, South Africa by the Curator Willem Froneman. The GPS coordinates for the harvested trees at the Lowveldt Botanical Gardens are provided in Table 1.

The leaves from each *Combretum* spp. leaves were air-dried in the shade at room temperature for approximately seven days until completely dry (as determined by consistent masses following further drying time). The dried leaves were then ground separately using a high speed Fritsch Pulverisette grinder (Labotec) to produce a coarse powder. One gram masses of each powdered leaf sample was extracted separately by maceration in 50 mL AR grade methanol (Ajax Fine Chemicals, Australia) and filtered through Whatman No. 54 filter paper to remove the particulate matter. The filtrates were subsequently air dried at room temperature in the shade and the mass of the resultant dried extracts were recorded to determine the extract yield (Table 1). The dried extracts were then resuspended in 10 mL deionised water (containing 1 % DMSO), and filtered through a 0.22 μm filter (Sarstedt) and stored at 4 °C until use.

2.2. Qualitative phytochemical studies

Qualitative phytochemical evaluations of the *Combretum* spp. leaf extracts to detect and evaluate the relative abundances of phenolic compounds, flavonoids and tannins were conducted using standard assays (Lee et al., 2016; Wright et al., 2016; Winnett et al., 2017).

2.3. Antibacterial analysis

2.3.1. Conventional antibiotics

Ampicillin (≥96 %), ciprofloxacin (≥98 %), gentamicin (600 μg/mg), methicillin (≥95 % purity), and oxacillin (≥95 % purity) were purchased from Sigma-Aldrich, Australia. All antibiotics were prepared as 0.01 mg/mL stocks in sterile deionised water and used as control antibiotics in the broth microdilution assay. For the disc diffusion screening studies, pre-loaded ampicillin (10 μg), Augmentin® (15 μg), cefoxitin (30 μg), chloramphenicol (10 μg), ciprofloxacin (5 μg), gentamicin (30 μg), methicillin (5 μg), oxacillin (5 μg) and tetracycline (10 μg) susceptibility discs (Oxoid Ltd., Australia) were used to screen the bacterial strains for antibiotic susceptibility, and as positive assay controls.

Table 1
The *Combretum* species used in this study, their collection site and gps coordinates (where available), the mass of dried extracted material, the concentration after resuspension in 1 % DMSO and qualitative phytochemical screenings for phenolics, flavonoids and tannins of the Combretum *spp.* extracts.

Extract	Common Name	Collection site	GPS Coordinates	Extraction Mass (g)	Extract Concentration (mg/mL)	Phenolics	Flavonoids	Tannins
Combretum bracteosum (Hochst.) Engl. & Diels	Hiccup nut	Walter Sisulu Botanical Gardens, Johannesburg, South Africa	NR	324	32.4	+++	++	+++
Combretum collinum Fresn.	Variable bushwillow, variable combretum	Lowveldt Botanical Gardens, Nelspruit, South Africa	25° 26′ 36.92" S 30° 58′ 12.31" E	388	38.8	+++	+++	+++
Combretum erythrophyllum (Burch.) Sond.	River bushwillow	Lowveldt Botanical Gardens, Nelspruit, South Africa	25° 26′ 35.63" S 30° 58′ 13.11" E	318	31.8	+++	++	+++
Combretum hereroense Schinz	Russet bushwillow, mouse-eared combretum	Lowveldt Botanical Gardens, Nelspruit, South Africa	25° 26′ 33.38" S 30° 58′ 12.73" E	242	24.2	+++	+++	+++
Combretum molle R.Br ex G. Don	Velvet bushwillow	Lowveldt Botanical Gardens, Nelspruit, South Africa	25° 26′ 35.54" S 30° 58′ 14.56" E	255	25.5	+++	+++	+++
Combretum vendae A.E.van Wyk	Venda bushwillow	Lowveldt Botanical Gardens, Nelspruit, South Africa	25° 26′ 37.33" S 30° 58′ 14.47" E	219	21.9	+++	+++	+++

NR = the gps coordinates were not provided; +++ indicates a large response; ++ indicates a moderate response.

2.3.2. Bacterial cultures

Methicillin-sensitive *Staphylococcus aureus* (ATCC 25923) and β-lactam sensitive *Escherichia coli* (ATCC 25922) and *Klebsiella pneumoniae* (ATCC 31488) were purchased from American Type Culture Collection, USA (ATCC) and used in this study. A reference strain of MRSA (ATCC 43300) was also included in this study. Extended spectrum β-lactamase (ESBL) resistant clinical isolate strains of *E. coli* and *K. pneumoniae* were kindly donated by Dr Matthew Cheesman of the School of Pharmacy and Medicinal Sciences, Griffith University and were originally obtained from Gold Coast University Hospital, Australia. All *E. coli* and *K. pneumoniae* strains were cultured using Mueller-Hinton agar or broth (Oxoid Ltd., Australia). The antibiotic-sensitive and MRSA *S. aureus* strains were grown using the same media supplemented with 2 % NaCl. All bacteria were cultured at 37 °C for 24 h, with the exception of the MRSA strain, which was maintained and tested at 35 °C. Streaked agar plates were tested in parallel throughout these experiments to ensure the purity of all bacterial cultures.

2.3.3. Evaluation of bacterial susceptibility to growth inhibition

Bacterial susceptibility to the *Combretum* spp. extracts and the conventional antibiotics was assessed by standard disc diffusion assays (Ilanko and Cock, 2019). Discs preloaded with ampicillin (10 μg), Augmentin® (15 μg), cefoxitin (30 μg), chloramphenicol (10 μg), ciprofloxacin (5 μg), gentamicin (30 μg), methicillin (5 μg), oxacillin (5 μg) and tetracycline (10 μg) were obtained from Oxoid Ltd., Australia and included in the assays as positive controls. Filter discs infused with 10 μL of distilled water were included as a negative control. Following an overnight incubation at 37 °C (or 35 °C for MRSA) on Mueller-Hinton agar plates, the zones of inhibition (ZOIs) were measured to the nearest whole mm. All tests were performed three times, each with three technical replicates (n = 9) and the results are presented herein as the means ± SEM. One-way ANOVA analysis was used to calculate statistical significance between control and treatment groups, with $P < 0.01$ classified as significant.

2.3.4. Minimum inhibitory concentration (MIC) determination

The antibacterial activity of the individual *Combretum* spp. leaf extracts and conventional antibiotics was quantified using standard liquid dilution MIC assays (Hűbsch et al., 2014 Ilanko et al., 2019; Tiwana et al., 2020). Briefly, log phase bacterial cultures (determined by optical density measurements) were diluted in broth to produce a 0.5 McFarland turbidity standard. Sterilized broth (100 μL) was dispensed into each well of 96 well micro-titre plate and 100 μL of the extracts or antibiotics were added separately to individual wells and diluted by two-fold serial dilutions down the individual columns of the plate. Mueller-Hinton broth (negative control) and a sterile control (media without bacteria) were included on all plates to verify that the assay was functioning correctly. Bacterial culture (100 μL containing approximately 1×10^6 colony forming units (CFU)/mL) was subsequently added to all wells of the plate (excluding the sterile control wells) and the plates incubated at 37°C (or 35 °C for the MRSA plates) for 24 h. *p*-Iodonitrotetrazolium violet (INT) colourimetric indicator (Sigma-Aldrich, Australia) was prepared as a 0.2 mg/mL solution in sterile deionised water and 40 μL of the INT stock solution was dispensed into all wells. The plates were incubated for 6 h at 30° C to allow full colour development. The MIC was classified as the lowest dose at which colour development was inhibited. To ensure the reliability of the calculated MIC values, each assay was repeated on separate days, with two technical replicates included on individual days (n = 4).

2.4. Toxicity screening

Toxicity of the *Combretum* spp. leaf extracts was evaluated using two toxicity assays. The *Artemia* nauplii lethality assay (ALA) was utilised to provide a rapid initial screening of the extracts, whilst a human dermal fibroblast (HDF) cell viability assay was used as a cellular evaluation of toxicity.

2.4.1. Artemia franciscana Kellogg nauplii lethality assay

The toxicity of the *Combretum* spp. leaf extracts was evaluated using *Artemia franciscana* nauplii lethality assays (ALA) adapted for plant extracts (Ruebhart et al., 2009). Dried *A. franciscana* cysts were purchased from Ocean Nutrition, USA and were hatched and assayed in Tropic Marine Salt (Australia) artificial seawater. Potassium dichromate (1 mg/mL; AR grade, Chem-Supply) and artificial seawater were used as a reference toxin and negative control respectively. All tests were evaluated across a range of concentrations and the LC_{50} values were calculated using Probit analysis.

2.4.2. Human dermal fibroblast (HDF) cellular viability assay

All *Combretum* spp. leaf extracts were all screened for 24 h at 200 μg/mL against normal human primary dermal fibroblasts (HDF; ATCC PCS-201-012; American Type Culture Collection, USA) and the % cell viability was calculated by standard colorimetric methods

(Shalom and Cock, 2018). Cell Titre 96 Aqueous One solution, containing MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt) was used as the indicator reagent). Absorbances were measured using a Molecular Devices, Spectra Max M3 plate reader at 540 nm (blank wavelength 690 nm). All tests were performed three times in triplicate (n = 9) and the results are reported as mean ± SEM. Triplicate controls of quinine (AR grade, Sigma Australia) as the positive control, and media as the negative control) were included on each plate to verify the validity of the assay. The % cellular viability of each test was calculated using the following formula:

$$\%\ \text{cellular viability} = \frac{\text{Abs test sample} - (\text{mean Abs control} - \text{mean Abs blank})}{(\text{mean Abs control} - \text{mean Abs blank})}$$

Cellular viability ≤50 % (compared to the untreated control) was classified as toxic, whilst cell viability >50 % untreated control were deemed to be non-toxic.

2.4.3. Evaluation of therapeutic index

The therapeutic indexes (TI) of the *Combretum* spp. leaf extracts were calculated using the following formula and are included as a measure of the suitability of the extracts for therapeutic usage:

$$\text{Therapeutic index} = (\text{ALA } LC_{50})/(\text{MIC})$$

2.5. GC-MS head space fingerprint analysis

Chromatographic separation, putative identification and determination of the relative abundance of targeted compounds was achieved using previously optimised methods (Nel et al., 2020) on a Shimadzu GC-2010 Plus (USA) gas chromatography system coupled to a Shimadzu MS TQ8040 (USA) mass selective detector system. Chromatographic separation was achieved using a 5 % phenyl, 95 % dimethylpolysiloxane capillary column (30m x 0.25 mm internal diameter), using high grade helium as the carrier gas. The mass spectrometer was operated in the electron ionisation mode at 70 eV and mass spectral data was recorded in total ion count (TIC) mode for a 45 min period across a 45-450 m/z mass range. Structural identity was determined by comparison with mass spectral data in the National Institute of Standards and Technology (NIST) database. The calculated retention index (RIs) of each compound were then compared to RI values reported in the NIST database and compounds with RI values outside that range were considered ambiguous and are not reported herein.

2.6. Statistical analysis

All data presented herein is expressed as the mean ± SEM of three independent experiments, each with internal triplicates (n = 9). One-way analysis of variance analysis (ANOVA) was used to calculate statistical significance between the negative control and treated groups, with a $P < 0.01$ considered to be statistically significant.

3. Results

3.1. Liquid extraction yields and qualitative phytochemical screening

Maceration of the powdered *Combretum* spp. leaves (1 g) in methanol yielded dried plant extracts ranging from 219 mg (*C. vendae* leaf extract) to 388 mg (*C. collinum* leaf extract) (Table 1). Qualitative phytochemical screening showed that the extracts of all *Combretum* spp. contained high relative abundances of total polyphenolics, flavonoids and tannins (Table 1).

3.2. Bacterial growth inhibition

3.2.1. Antibiotic susceptible and methicillin-resistant Staphylococcus aureus strains

The *Combretum* spp. methanolic leaf extracts were screened against reference strains of antibiotic sensitive *S. aureus* (Fig. 1a) and a methicillin-resistant strain (MRSA) (Fig. 1b) to evaluate the antimicrobial potential. The antibiotic resistance was verified by screening these bacteria against a variety of clinical antibiotic therapies. As expected, the ATCC 25923 reference strain of *S. aureus* was highly susceptible to methicillin, as well as all of the other β-lactam antibiotics tested (ampicillin, Augmentin®, cefoxitin, oxacillin), with ZOIs generally >22 mm (Fig. 1a). Notably, this *S. aureus* strain was also highly susceptible to the other non-β-lactam antibiotics, with ZOIs between 16.2 and 23.1 mm. In contrast, the MRSA strain (ATCC 43300) was resistant to methicillin, with only a 7.2 mm ZOI measured (Fig. 1b). Furthermore, this strain was completely resistant to oxacillin and had reduced sensitivity to all other β-lactam antibiotics compared to the antibiotic sensitive (ATCC 25923) strain. Notably, the susceptibility of the MRSA strain towards the non-β-lactam antibiotics was also substantially decreased compared to the antibiotic sensitive strain, which makes infections of this strain particularly difficult to treat. Indeed, the MRSA strain was completely resistant to gentamicin in the disc diffusion susceptibility assay, and substantially reduced ZOIs were measured for chloramphenicol and ciprofloxacin. Only tetracycline was as potent towards the MRSA strain as it was to the ATCC 25923 (antibiotic sensitive) strain.

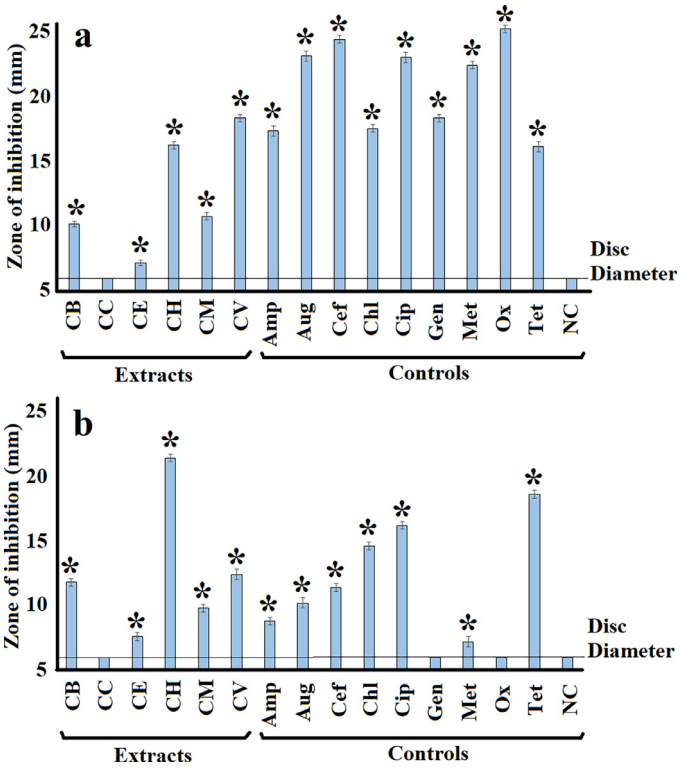

Fig. 1. Antibacterial activity of the *Combretum* spp. methanolic extracts against (a) *S. aureus* (ATCC 25923) and (b) MRSA (ATCC 43300) measured as zones of inhibition (mm). CE = *C. bracteosum*, CC = *C. collinum*; CE = *C. erythrophyllum*; CH = *C. hereroense*; CM = *C. molle*; CV = *C. vendae*; Amp = ampicillin (10 μg); Aug = Augmentin® (15 μg); Cef = cefotoxin (30 μgg); Chl = chloramphenicol (10 μg); Cip = ciprofloxacin (5 μg); Gen = gentamicin (30 μg); Met = methicillin (5 μg); Ox = oxacillin (5 μg); Tet = tetracycline (10 μg); NC = negative control (1 % DMSO). Results are expressed as mean zones of inhibition of three replicates, each with internal triplicated (n = 9) ± SEM.; * indicates results that are significantly different to the negative control ($P < 0.01$).

With the exception of *C. collinum*, all *Combretum* spp. extracts inhibited the growth of both the antibiotic-sensitive and antibiotic-resistant *S. aureus* strains, although only small ZOIs were measured against *C. erythrophyllum*, indicating relatively weak activity for that extract. The growth inhibitory activity of the *C. hereroense* extract was particularly noteworthy, with a ZOI of 21.4 mm recorded against the MRSA strain. Interestingly, the MRSA strain was substantially more sensitive to the methanolic *C. hereroense* leaf extract than the antibiotic-sensitive strain was, indicating that the extracts may be particularly useful for the treatment of MRSA infections. Furthermore, antibiotic compounds in the extract may work by substantially different mechanisms to methicillin. It may therefore be advantageous to use combinations of methicillin and the *C. hereroense* extract (or ideally, isolated extract components), to not only enhance the antibiotic efficacy of the treatment, but also to decrease the possibility of bacteria developing further resistance. Notably, previous studies have reported a similar trend, with the same MRSA strain being more sensitive to *Terminalia* spp. extracts than an antibiotic-sensitive *S. aureus* strain (Cheesman et al., 2019). Whilst inducing substantially smaller ZOIs than the *C. hereroense* leaf extract, the *C. bracteosum, C. molle* and *C. vendae* methanolic leaf extracts also inhibited the growth of both strains substantially, and generally to similar extents.

3.2.2. Antibiotic susceptible and ESBL Escherichia coli strains

The growth inhibitory activity of the conventional antibiotics was also tested against the antibiotic-sensitive (Fig. 2a) and ESBL strains of *E. coli* (Fig. 2b). Not surprisingly, large ZOIs were measured when the majority of the β-lactam antibiotics were screened against the β-lactam sensitive *E. coli* strain (Fig. 2a). Indeed, ZOIs of 10.2, 17.8 and 26.3 mm were measured for ampicillin, Augmentin® and cefotoxin respectively against that bacterium. Interestingly, oxacillin was completely ineffective against the reference *E. coli* strain and only small ZOIs (6.6 mm) were measured for methicillin. In contrast, substantially smaller ZOIs were measured for ampicillin and Augmentin® against the ESBL strain (Fig. 2b) than for the β-lactam sensitive *E. coli* strain, confirming the resistance of that strain to β-lactam antibiotics. Whilst the ESBL strain was still strongly inhibited by cefotoxin (ZOI = 23.2 mm), it was substantially less potent than it was against the β-lactam sensitive *E. coli* strain (ZOI = 26.3 mm). Interestingly, as reported for the bacterial *S. aureus* pair, substantially larger ZOIs were measured for methicillin and oxacillin against the ESBL *E. coli* strain (11.3 and 7.7 mm respectively) than for the β-lactam sensitive *E. coli* strain (6.6 mm and completely ineffective respectively).

Several of the *Combretum* spp. extracts were also good inhibitors of the growth of both β-lactam sensitive and ESBL *E. coli* strains. Similar to the trend noted for the *S. aureus* bacterial pair, the methanolic *C. hereroense* leaf extract was the strongest inhibitor of *E. coli* growth. Indeed, ZOIs of approximately 20 mm were measured when the *C. hereroense* extract was screened against the β-lactam sensitive *E. coli* strain (Fig. 2a). This compares favourably against the antibiotic controls. Indeed, the ZOI for this extract was larger than recorded for all of the antibiotic controls except cefotoxin and ciprofloxacin. Notably, the antibiotic controls are pure compounds, whereas the extract is a crude mixture, of which the antibiotic component(s) may account for a relatively low percentage of the total mass of extracted compounds. It is therefore likely that the activity of the individual extract components may be substantially greater than that of the antibiotic controls and further investigation is required to test that hypothesis. The *C. hereroense* leaf extract was also the best inhibitor of the ESBL *E. coli* strain (Fig. 2b), although with substantially smaller ZOIs (15.8 mm). The *C. molle* leaf extract also strongly inhibited the growth of the β-lactam sensitive and ESBL *E. coli* strains, with ZOIs of approximately 13 mm against both strains. All other *Combretum* spp. extracts also inhibited the growth of both *E. coli* strains with similar potency, although with substantially smaller ZOIs measured (8-10 mm).

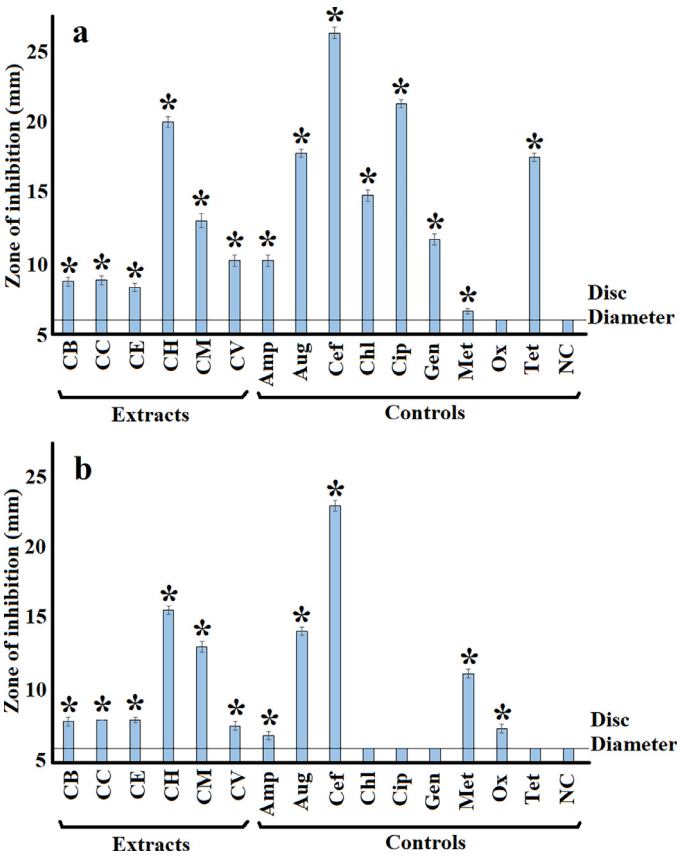

Fig. 2. Antibacterial activity of the *Combretum* spp. methanolic extracts against (a) *E. coli* (ATCC 25922) and (b) ESBL *E. coli* (clinical isolate strain) measured as zones of inhibition (mm). CE = *C. bracteosum*, CC = *C. collinum*; CE = *C. erythrophyllum*; CH = *C. hereroense*; CM = *C. molle*; CV = *C. vendae*; Amp = ampicillin (10 μg); Aug = Augmentin® (15 μg); Cef = cefotoxin (30 μgg); Chl = chloramphenicol (10 μg); Cip = ciprofloxacin (5 μg); Gen = gentamicin (30 μg); Met = methicillin (5 μg); Ox = oxacillin (5 μg); Tet = tetracycline (10 μg); NC = negative control (1 % DMSO). Results are expressed as mean zones of inhibition of three replicates, each with internal triplicated (n = 9) ± SEM.; * indicates results that are significantly different to the negative control ($P < 0.01$).

3.2.3. Antibiotic susceptible and ESBL Klebsiella pneumoniae strains

The growth of the β-lactam sensitive *K. pneumoniae* reference strain (ATCC 31488) was also substantially inhibited by all of the β-lactam antibiotics (Fig. 3a). Augmentin® and cefotoxin were particularly strong inhibitors of that bacterial strain, with ZOIs of 15.2 and 19.3 mm respectively. Noteworthy, albeit smaller ZOIs, were also measured for ampicillin (7.6 mm), methicillin (8.2 mm) and oxacillin (10.6 mm). This strain was also susceptible to the non-β-lactam antibiotics chloramphenicol (ZOI = 7.6 mm), ciprofloxacin (ZOI = 11.2 mm), gentamicin (ZOI = 10.4 mm) and tetracycline (ZOI = 10.4mm). In contrast, the ESBL *K. pneumoniae* strain was resistant to most of the antibiotics tested (Fig. 3b), confirming the β-lactam resistant status of this strain. Indeed, the ESBL strain was completely unaffected by ampicillin. Whilst the other β-lactam antibiotics inhibited the growth of that bacterium, the effects were substantially reduced compared to the β-lactam sensitive *K. pneumoniae* reference strain, with ZOIs of 7.6 and 9.6 mm measured for methicillin and oxacillin respectively. However, the growth of the ESBL *K. pneumoniae* strain was inhibited strongly by Augmentin® and cefotoxin, with ZOIs similar to those measured for the β-lactam sensitive *K. pneumoniae* reference strain (15.6 and 18.8 mm respectively). Interestingly, Augmentin® and cefotoxin differ from the other β-lactam antibiotics studied herein. Augmentin® is a mixture of two compounds with β-lactam structures. The amoxicillin component contributes the antibiotic potential of the therapy, whilst clavulanic

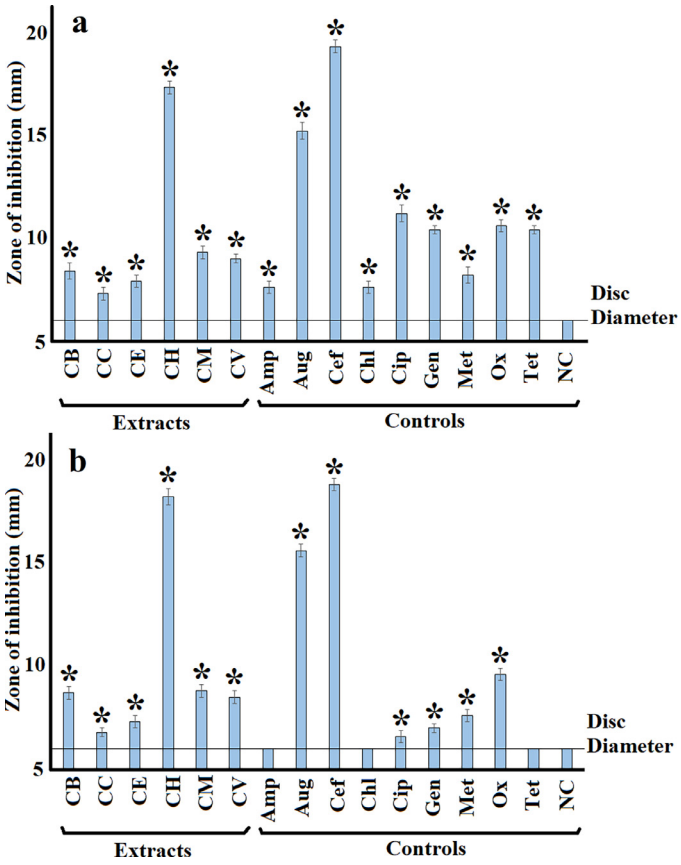

Fig. 3. Antibacterial activity of the *Combretum* spp. methanolic extracts against (a) *K. pneumoniae* (ATCC 31488) and (b) ESBL *K. pneumoniae* (clinical isolate strain) measured as zones of inhibition (mm). CE = *C. bracteosum*, CC = *C. collinum*; CE = *C. erythrophyllum*; CH = *C. hereroense*; CM = *C. molle*; CV = *C. vendae*; Amp = ampicillin (10 μg); Aug = Augmentin® (15 μg); Cef = cefotoxin (30 μgg); Chl = chloramphenicol (10 μg); Cip = ciprofloxacin (5 μg); Gen = gentamicin (30 μg); Met = methicillin (5 μg); Ox = oxacillin (5 μg); Tet = tetracycline (10 μg); NC = negative control (1 % DMSO). Results are expressed as mean zones of inhibition of three replicates, each with internal triplicated (n = 9) ± SEM.; * indicates results that are significantly different to the negative control ($P < 0.01$).

acid binds irreversibly to bacterial ESBL enzymes, thereby inactivating them and allowing amoxicillin to function again, even in β-lactam-resistant bacteria. It is likely that Augmentin® functions strongly in the ESBL *K. pneumoniae* strain because clavulanic acid inactivates the bacteria's resistance mechanism. In contrast, cefotoxin is a relatively new antibiotic and many bacterial ESBL's have not yet adapted resistance to it. Notably, the ESBL *K. pneumoniae* strain was also resistant to all of the other (non-β-lactam) antibiotics tested. Indeed, chloramphenicol and tetracycline were completely ineffective against this strain in the disc diffusion assay, whilst relatively small ZOIs were recorded for ciprofloxacin (6.6 mm) and gentamicin (7 mm). Thus, infection of the ESBL *K. pneumoniae* strain would be particularly difficult to treat and alternative chemotherapies are required.

Similar susceptibility profiles were noted for both *K. pneumoniae* strains against the *Combretum* spp. leaf extracts. The methanolic *C. hereroense* leaf extract was particularly strong inhibitor of both strains, with ZOIs of 17.3 and 18.2 mm measured for the β-lactam sensitive *K. pneumoniae* reference strain and the ESBL strain respectively. Thus, the *C. hereroense* extract is particularly promising for the treatment of *K. pneumoniae* infections. Noteworthy activity was also noted for all other *Combretum* spp. extracts, albeit with substantially smaller ZOIs (generally between 8 and 9 mm). The similar potency of the *Combretum* spp. extract against the β-lactam sensitive and resistant *K. pneumoniae* strains indicates that it is unlikely that they function via inactivation of ESBL enzymes. Instead, it is likely the extracts contain compounds that may function via other mechanisms (e.g. inhibition of bacterial efflux pumps). Substantially more work is required to elucidate the antibacterial mechanisms of the *Combretum* spp. leaf extracts and future studies are planned to examine these mechanisms. However, the activity of the extracts against both antibiotic sensitive and resistance strains indicates that these extracts (especially the methanolic *C. hereroense* leaf extract) may be particularly useful to treat *K. pneumoniae* infections.

3.3. Quantification of minimum inhibitory concentration (MIC)

The antimicrobial activity of the methanolic *Combretum* spp. leaf extracts and conventional antibiotics was further evaluated by determining the MIC values using liquid dilution MIC assays, to allow for comparisons with other studies and to benchmark the activity between extracts (Table 2). Notably, MIC values >1 μg/mL have previously been defined as demonstrating antibiotic resistance for pure antibiotics (Hűbsch et al., 2014; Ilanko et al., 2019; Tiwana et al., 2020). Using that definition, both the *E. coli* and *K. pneumoniae* ESBL strains were resistant to all of the antibiotics tested, whilst the MRSA strain was resistant to all antibiotics except ciprofloxacin (MIC = 0.4 μg/mL). In contrast the methicillin-sensitive *S. aureus*

Table 2
MIC values of the *Combretum* spp. extracts and conventional antibiotics (μg/mL) against some bacterial triggers of selected autoimmune anti-inflammatory diseases.

Extract or Antibiotic		*Staphylococcus aureus*		*Escherichia coli*		*Klebsiella pneumoniae*	
		(ATCC 25923)	MRSA	(ATCC 25922)	ESBL strain	(ATCC 31488)	ESBL strain
Extracts	**CB**	2000	500	1000	500	1000	500
	CC	490	490	250	490	250	120
	CE	1625	1625	1625	813	1625	1625
	CH	340	170	340	340	340	680
	CM	890	445	890	890	890	223
	CV	115	230	230	460	460	460
Conventional Antibiotics	**Amp**	0.63	2.5	0.39	>2.5	0.63	>2.5
	Cip	0.4	0.4	0.01	>2.5	0.31	>2.5
	Gent	0.8	>2.5	0.16	>2.5	0.63	>2.5
	Met	0.31	1.56	>2.5	>2.5	>2.5	>2.5
	Ox	0.04	2.5	>2.5	>2.5	>2.5	>2.5

MRSA = methicillin-resistant S. aureus; ESBL = extended spectrum β-lactamase resistant strain; CE = *C. bracteosum*, CC = *C. collinum*; CE = *C. erythrophyllum*; CH = *C. hereroense*; CM = *C. molle*; CV = *C. vendae*; Amp = ampicillin; Cip = ciprofloxacin; Gent = gentamycin; Met = methicillin; Ox = oxacillin.

strain was sensitive to all of the antibiotics quantified in the MIC assay, whilst the reference and β-lactam sensitive *E. coli* and *K. pneumoniae* strains were resistant to methicillin and oxacillin, but were sensitive to the other reference antibiotics.

The methanolic *Combretum* spp. extracts displayed noteworthy growth inhibitory activity against all of the bacterial strains tested (Table 2). The MIC quantification results are generally consistent with the disc diffusion susceptibility testing results. The methanolic *C. hereroense* leaf extract was a particularly good inhibitor of all bacterial strains (MICs = 170-680 μg/mL). Interestingly, the *C. hereroense* extract was more a potent inhibitor of the MRSA resistant strain than against the β-lactam sensitive reference *S. aureus* strain, which is consistent with the trend noted for the disc diffusion susceptibility assays. In contrast, the *C. hereroense* leaf extract inhibited the β-lactam sensitive and ESBL strain of *E. coli* and *K. pneumoniae* with approximately equal potency (as judged by MIC). The *C. vendae* leaf extract was also a good inhibitor of all bacterial strains tested, although it was a particularly good inhibitor of the β-lactam sensitive strains (MICs of 115, 230 and 460 μg/mL against the β-lactam sensitive strains of *S. aureus, E. coli* and *K. pneumoniae* respectively). The *C. vendae* leaf extract was also a good inhibitor of the corresponding MRSA and ESBL strains, albeit with MIC values that were generally twice the MICs measured against the β-lactam antibiotic sensitive strains. The *C. collinum* and *C. molle* leaf extracts were also good inhibitors of all bacterial strains tested, with MIC values generally 120-890 μg/mL. Whilst the methanolic *C. bracteosum* and *C erythrophyllum* leaf extracts also had noteworthy growth inhibitory activity against the bacterial panel, substantially higher MIC values were recorded against the β-lactam sensitive bacterial strains (1000-2000 μg/mL) than for the MRSA and ESBL strains (500-1625 μg/mL).

3.4. Toxicity studies

All of the *Combretum* spp. leaf extracts were initially serially diluted and tested across a range of concentrations in the *Artemia* nauplii lethality assay (ALA) to allow for an evaluation of toxicity by calculating the LC_{50} values (Table 3). LC_{50} values substantially >1000 μg/mL were calculated for all *Combretum* spp. extracts. As LC_{50} values >1000 μg/mL are defined as non-toxic for crude extracts in this assay (Ruebhart et al., 2009; Courtney et al., 2015), all of the *Combretum* spp. extracts were deemed to be non-toxic. In contrast, an LC_{50} of 52 μg/mL was determined for the potassium dichromate control, confirming that the assay was functioning correctly. The extracts were also tested for their effects on cell viability in HDF cellular viability assays as cellular assays may provide a more relevant understanding of the toxicity in humans. As none of the *Combretum* spp. extracts decreased cell viability by ≥50 %, all were verified to be non-toxic to HDFs. In contrast, exposure to the positive control (quinine) reduced HDF cell viability by approximately 75 %, confirming that the assay was functioning correctly. Therefore, all of the methanolic *Combretum* spp. leaf extracts were deemed to be non-toxic in both toxicity assays.

3.5. Calculation of therapeutic index

To further evaluate the suitability of the *Combretum* spp. leaf extracts as therapeutic agents, their therapeutic indexes (TI) were calculated (Table 3). For this study, TI values ≥3 were considered noteworthy. The safety of the *C. collinum, C. hereroense* and *C. vendae* extracts was determined to be particularly promising, with TI values >3 against all of the bacteria tested for each of these extracts. Indeed, TI values of 15.3, 12.45 and 15.54 respectively were calculated for the *C. collinum* extract against the ESBL *K. pneumoniae* strain, *C. hereroense* against MRSA and the *C. vendae* extract against the β-lactam sensitive *S. aureus* reference strain. Therefore these extracts were considered to be safe to use therapeutically *in vitro*, although further *in vivo* studies are required to confirm the safety of these extracts before they can be adapted for clinical use.

3.6. GC-MS head space analysis

As volatile terpenoid components have previously been reported to contribute to the antibacterial activity of several *Combretum* and *Terminalia* spp. leaf extracts (Sirdaarta et al., 2015; Shalom and Cock, 2018), we selected GC-MS headspace analysis to identify volatile terpenoid components in the *Combretum* spp. extracts. Furthermore, recent reports have identified several monoterpenoid components in *Combretum* and *Terminalia* spp. leaves using similar GC-MS headspace analyses (; Sirdaarta et al., 2015; Courtney et al., 2015; Shalom and Cock, 2018). Specifically, borneol, camphor, cineole, limonene and terpineol have been identified in relative abundance in leaf extracts produced from multiple *Combretum* and *Terminalia* spp. extracts. Additionally, isomenthol and isomyocorene have also identified in some (but not all) species. When detected in other Combretaceae spp., these compounds were generally in lower relative abundances than the other identified monpoterpenoids. Our study targeted these compounds using GC-MS headspace analysis and quantified the relative abundance in the extracts of each compound identified (Table 4). Borneol (0.74-1.32 % relative abundance), camphor (0.27-0.48 % relative abundance), cineole (1.53-2.26 % relative abundance), limonene (0.33-0.46 % relative abundance), and terpineol (1.26-1.66 % relative abundance) were identified in relative

Table 3
Toxicity (expressed as LC_{50} in μg/mL), and the therapeutic index (TI) for the extracts and positive controls.

Extract	Toxicity (LC_{50} in μg/mL)		Therapeutic index					
	ALA	HDF	*S. aureus* (ATCC 25923)	MRSA	*E. coli* (ATCC 25922)	ESBL *E. coli*	*K. pneumoniae* (ATCC 31488)	ESBL *K. pneumoniae*
CB	2485	NT	1.24	*4.97*	2.49	*4.97*	2.49	*4.97*
CC	1840	NT	***3.76***	***3.76***	***7.36***	***3.76***	***7.36***	***15.3***
CE	2012	NT	1.24	1.24	1.24	2.48	1.24	1.24
CH	2117	NT	***6.23***	***12.45***	***6.23***	***6.24***	***6.23***	***3.11***
CM	1925	NT	2.16	***4.33***	2.16	2.16	2.16	***8.63***
CV	1787	NT	***15.54***	***7.77***	***7.77***	***3.88***	***3.88***	***3.88***
PC	52	34.7 ± 2.4*	ND	ND	ND	ND	ND	ND

Results represent means of 3 independent experiments, each preformed in triplicate (n = 9).; ALA = *Artemia* lethality assay; HDF = human dermal fibroblast toxicity assay; MRSA = methicillin-resistant *S. aureus*; ESBL = extended spectrum β-lactamase resistant strain; CE = *C. bracteosum*, CC = *C. collinum*; CE = *C. erythrophyllum*; CH = *C. hereroense*; CM = *C. molle*; CV = *C. vendae*; PC = positive control (potassium dichromate); NT = not toxic (>50 % viability) at all doses tested; ND = could not determined as the toxicity PC was not tested against the bacteria or the MIC was >5000 μg/mL; * = % cell viability rather than (LC_{50}). Notable TI values >3 are presented in bold and italics.

Table 4
Qualitative GC-MS headspace analysis of the *Combretum* spp. methanolic leaf extracts, elucidation of empirical formulas, putative identification of the compounds and determination of relative abundance.

Retention time (min)	Empirical formula	Molecular Mass (Da)	Compound identified	Relative Abundance (%)					
				C. bracteosum	*C. collinum*	*C. erythrophyllum*	*C. hereroense*	*C. molle*	*C. vendae*
14.09	$C_{10}H_{16}$	136	Limonene	0.33	0.42	0.46	0.34	0.35	0.41
14.58	$C_{10}H_{16}$	136	Isomyocorene	0.09	0.05	0.04	0.1	0.05	0.05
15.5	$C_{10}H_{18}O$	154	Cineole	1.53	2.2	2.26	1.83	1.87	1.84
17.71	$C_{10}H_{16}O$	152	Camphor	0.27	0.39	0.44	0.46	0.48	0.43
18.41	$C_{10}H_{18}O$	154	Borneol	0.74	1.08	1.25	1.14	1.32	1.27
19.16	$C_{10}H_{18}O$	154	Terpineol	1.26	1.58	1.66	1.38	1.38	1.47
39.46	$C_{10}H_{20}O$	165	Isomenthol	0.17	0.07	0.05	0.08	0.04	0.04

The relative abundance expressed in this table is a measure of the area under the peak expressed as a % of the total area under all chromatographic peaks.

abundance in all of the *Combretum* spp. leaf extracts, whilst isomenthol (0.04-0.17 % relative abundance) and isomyocorene (0.04-0.1 % relative abundance) were detected in substantially lower relative abundance. Interestingly the composition and relative abundances of these monoterpenoids are similar to the levels previously reported in other *Combretum* and *Terminalia* spp. using similar analytical methods (Wright et al., 2019).

4. Discussion

This study evaluated and quantified the growth inhibitory properties of selected southern African *Combretum* spp. leaf extracts against ESBL and methicillin-resistant bacterial pathogens, and compared the inhibition to the activity of the extracts against β-lactam sensitive bacterial strains. Notably, the antibiotic-resistant bacteria included in this study were previously been reported to be resistant to multiple β-lactams (Cheesman et al., 2019), which was confirmed in our study. However, we also screened these bacteria against an extended panel of antibiotics of multiple classes and determined that these pathogens were also resistant to multiple other classes of antibiotics. The ESBL *E. coli* and *K. pneumoniae* strains displayed particularly broad-spectrum antibiotic-resistance. Indeed, these strains were determined to be resistant to all of the tested antibiotics (as judged by MIC values), and only cefotoxin and Augmentin® strongly inhibited the growth of those strains in the disc diffusion susceptibility assays. The MRSA strain tested in our study also had broad-spectrum antibiotic resistance, although it was sensitive to ciprofloxacin.

Combretum spp. were selected for screening in our study as their antibacterial properties have are well established (Cock et al., 2022). One study screened extracts prepared from 22 South African *Combretum* spp. against a panel of bacterial pathogens and reported that *C. molle, C. petrophyium, C. moggi, C. erythrophyllum, C. padoides, C. paniculatum* and *C. mossambicense* had noteworthy inhibitory activity against *S. aureus, E. coli, E. faecalis* and *P. aeruginosa* in broth microdilution assays (Eloff, 1999). The *C. molle* extracts possessed the most promising activities in that study, with the lowest MIC values. Another study reported good antibacterial activity for several African *Combretum* spp., including the southern African species *C. hereroense* and *C. molle* in solid phase agar diffusion assays against *S. aureus, Enterobacter aerogenes, Staphylococcus epidermidis, Bacillus subtilis* and *Mycobacterium luteus* (Fyhrquist et al., 2002). A more recent study screened *C. collinum, C. erythrophyllum, C. hereroense, C. microphyllium* and *C. molle* leaf extracts against an extended panel of pathogenic bacterial strains, including *B. cereus, B. subtilis* and *S. aureus,* and reported potent broad-spectrum antibacterial activity, with MICs generally between 200 and 500 μg/mL (Cock and Van Vuuren, 2015). Notably, all of those studies have evaluated the antibacterial activity of the extracts against bacterial strains that are relatively sensitive to common clinically used antibiotics, including β-lactams. However, the development of widespread resistance to β-lactam antibiotics in recent years has substantially reduced the efficacy of this class of antibiotics clinically and the development of novel antibiotic chemotherapies against these resistant strains is urgently required. This study was undertaken to extend the earlier studies by evaluating southern African *Combretum* spp. extracts against multi-antibiotic resistant bacterial strains.

Noteworthy growth inhibitory activity was noted for all *Combretum* spp. extracts all of the bacterial strains tested (both antibiotic-sensitive and antibiotic-resistant strains), although the *C. hereroense* leaf extract had particularly good antibacterial activity against all bacterial strains (MICs 170-680 μg/mL). Notably, this extract was more potent against the methicillin-resistant *S. aureus* strain (MRSA) than against the antibiotic-sensitive reference *S. aureus* strain, indicating that extracts compounds may be functioning via different mechanisms to methicillin. Alternatively (or additionally), *C. hereroense* leaf extract components may be potentiating the activity of other components, possibly by blocking the resistance mechanism(s) of this bacterium. If this is indeed the case, these findings could have substantial therapeutic applications as it may indicate that some extract components may allow methicillin (and other β-lactam antibiotics) to function effectively again, even against bacterial strains otherwise resistant to their effects. This would not only increase the efficacy of those antibiotics, but would also extend their useful lifespan. Future studies are required to screen the activity of these extracts in combination with conventional antibiotics, and if justified, to isolate the activity potentiating compounds and determine their potentiation mechanism(s). Similarly, the *C. vendae* extract was also a good inhibitor of the antibiotic-resistant MRSA and ESBL strains. Noteworthy bacterial growth inhibitory activity was also noted for *C. collinum* and *C. molle* extracts against all bacterial strains (MIC values generally between 120 and 890 μg/mL). In contrast, the *C. bracteosum* and *C erythrophyllum* extracts had substantially higher MIC values were recorded against the β-lactam sensitive bacterial strains (1000-2000 μg/mL) than for the MRSA and ESBL strains (500-1625 μg/mL).

The GC-MS headspace studies presented herein highlighted the relative abundance of volatile monoterpenoid components in all of the *Combretum* spp. extracts. Notably, several monterpenoids (including several identified in our study) have substantial antibacterial activity (Guimarães et al., 2019). Cineole and terpineol were present in particular abundance in all of the *Combretum* spp. extracts (1.2-2.3 % relative abundance). Interestingly, cineole has been reported to have noteworthy antibacterial activity by modulating the fluidity of the bacterial membrane (Moghimi et al., 2017). That study reported MIC values substantially below 1 mg/mL. Terpineol derivatives have also been reported to inhibit the growth of several bacteria, with similar MIC values (Huang et al., 2021). Notably, that study also reported that the terpineol derivatives also induced morphological changes in the tested bacteria, so it is likely that terpineol and cineole function via similar mechanisms. Limonene, camphor and borneol were also present in all of *Combretum* spp. extracts in substantial (albeit lower) levels. These monoterpenoids also

have noteworthy antibacterial activity (Guimarães et al., 2019). Thus it is likely that the identified monoterpenoids contribute to the antibacterial activity of the *Combretum* spp. extracts, although this remains to be verified.

Notably, whilst the headspace GC-MS method used in our study was useful for identification of volatile components, it will not identify larger less volatile low polarity compounds. Further phytochemical analysis using high resolution GC-MS is required to identify those components. Similarly, headspace GC-MS will also not identify many high polarity compounds, and future studies using high resolution LC-MS are also required to obtain a more complete understanding of the composition of these extracts. Additionally, whilst GC-MS is useful as a qualitative tool, it lacks the sensitivity required for quantification of the levels of the individual compounds in the extracts. Indeed, we have only reported % relative abundances in our study. For more quantitative and reproducible results, future studies should also evaluate the extracts using GC-FID.

It is likely that other phytochemical classes may also contribute to the antibacterial activity of the *Combretum* spp. extracts reported herein. Indeed, high tannin contents are a characteristic feature of *Combretum* spp. and have been reported for several southern African *Combretum* spp., with gallic acid and ellagic acid, being particularly well reported (Jossang et al., 1994; Adnyana et al., 2001; Grønhaug et al., 2008; Roy et al., 2014; Cock et al., 2022). Notably, potent antibacterial activity has been reported for multiple tannins against a broad-spectrum of bacteria (Hogg and Embery, 1982; Wolinsky and Sota, 1984; Buzzini et al., 2008; Cock, 2015). Gallotannins (including gallic acid) bind to and precipitate bacterial cell surface proteins, thereby rendering them nonfunctional (Hogg and Embery, 1982; Buzzini et al., 2008), as well as inhibiting intracellular glucosyltransferase enzymes (Wu-Yuan et al., 1988). Ellagitannins are also potent inhibitors of bacterial growth and function via multiple mechanisms including modulating bacterial redox status, and by disrupting bacterial cell walls (Buzzini et al., 2008). Additionally, high flavonoid contents have also been reported for multiple *Combretum* spp. (Martini et al., 2004; Fankam et al., 2015; Kulawe et al., 2019; Cock et al., 2022). Many studies have reported potent antibacterial activities for a wide variety of flavonoids via a variety of mechanisms, including by binding to and inactivating bacterial cell wall proteins and modulating cellular redox status by inactivating intracellular oxidoreductases (Kaur and Arora, 2009; Narayana et al., 2001). The antibacterial properties of ellagitannins has also been attributed to a variety of other mechanisms, including their ability to complex with extracellular and soluble proteins, as well as disrupting bacterial cell walls (Kaur and Arora, 2009). It is likely that tannins and flavonoids also contribute to the antibacterial activities of the southern African *Combretum* spp. reported herein.

Notably, none of the *Combretum* spp. extracts tested in this study were toxic in the ALA or HDF toxicity assays. Furthermore, noteworthy TI values were calculated for several extracts. Indeed, TI values substantially >3 were determined for *C. collinum, C. hereroense* and *C. vendae* against all of the bacterial strains tested. The calculated TI values for *C. collinum* extract against ESBL *K. pneumoniae* (15.3), and *C. vendae* against the antibiotic-sensitive *S. aureus* strain (15.54) indicate that these extracts are particularly safe for therapeutic usage. However, further *in vitro* toxicity studies using other human cell lines are required to verify the safety of these extracts before they are adapted for clinical usage. Future studies should also use *in vivo* toxicity assays to confirm the safety of these extracts (and isolated compounds) in complex biological systems.

5. Conclusions

The findings reported herein indicate the potential of selected southern African *Combretum* spp. extracts to inhibit selected β-lactam resistant strains of *E. coli, K. pneumoniae* and *S. aureus*. All *Combretum* spp. extracts displayed noteworthy inhibitory activity against antibiotic sensitive and resistant strains of *E. coli*. Similarly, all extracts except the *C. erythrophyllum* extract had noteworthy activity against *K. pneumoniae* and *S. aureus* (both antibiotic-sensitive and antibiotic-resistant strains). Furthermore, our study also determined the extracts to be non-toxic and highlighted several notable terpenoids that may contribute to the antibacterial activity of the extracts. Further studies to evaluate the therapeutic mechanisms of the extracts are warranted.

Declaration of Competing Interest

All authors declare that they have no competing interests.

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