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RESEARCH ARTICLE

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# Bioautography-guided HPTLC–MS as a rapid hyphenated technique for the identification of antimicrobial compounds from selected South African Combretaceae species

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## Abstract

**Introduction:** Many species within Combretaceae are traditionally used for the treatment of bacterial infections. The similarity in chemistry and antimicrobial activities within the family pose a challenge in selecting suitable species for herbal drug development.

**Objective:** This study aimed at rapidly identifying antimicrobial compounds using bioautography-guided high-performance thin-layer chromatography coupled with mass spectrometry (HPTLC–MS).

**Methods:** Hierarchical cluster analysis of ultra-performance liquid chromatography-mass spectrometry data from the methanol extracts of 77 samples, representing four genera within Combretaceae, was carried out. Based on groupings on the dendrogram, 15 samples were selected for bioautography analysis against four pathogens (*Staphylococcus aureus*, *Bacillus cereus*, *Escherichia coli* and *Salmonella typhimurium*). Active compounds were identified using HPTLC–MS analysis of bands corresponding to the inhibition zones.

**Results:** Bioautography revealed 15 inhibition zones against the four pathogens, with the most prominent present for *Combretum imberbe*. Analysis of the active bands, using HPTLC–MS indicated that flavonoids, triterpenoids and combretastatin B5 contributed to the antibacterial activity. The compounds corresponding to molecular ions *m/z* 471 (*Combretum imberbe*) and 499 (*Combretum elaeagnoides*) inhibited all four pathogens, and were identified as imberbic acid and jessic acid, respectively. Chemotaxonomic analysis indicated that arjunic acid, ursolic acid and an unidentified triterpenoid (*m/z* 471) were ubiquitous in the Combretaceae species and could be responsible for their antibacterial activities.

**Conclusion:** Application of HPTLC–MS enabled the rapid screening of extracts to identify active compounds within taxonomically related species. This approach allows for greater efficiency in the natural product research workflow to identify bioactive compounds in crude extracts.

KEYWORDS
antimicrobial, bioautography, Combretaceae, HPTLC–MS

## 1 | INTRODUCTION

Species belonging to the family Combretaceae are traditionally used to treat conditions associated with microbial infections.[1,2] Several studies have reported on the antimicrobial potential of crude organic and aqueous extracts of different species belonging to Combretaceae against bacterial and fungal pathogens.[3,4] Bioactivity-guided fractionation has previously demonstrated the successful isolation of flavonoids,[5,6] combretastatins,[7] phenanthrenes,[8] lignans,[9,10] triterpenoids,[11] phenolic acids,[12] and tannins[13] from various genera of Combretaceae. These classes of compounds have been reported to possess antimicrobial activities that contribute to the efficacy of these species in the management of microbial infections. A study of Eldeen *et al.*[10] indicated that lignans and triterpenoids contributed to the antibacterial activity of *Terminalia sericea*, while Anokwuru *et al.*[13] indicated that tannins and triterpenoids contributed to the activity of the same species. The triterpenoid, imberbic acid, was the main antibacterial agent in *Combretum imberbe*,[14,15] while combretastatin B5 (a stilbene) was the main antibacterial agent in *Combretum woodii*.[7] A sensitive and quick microplate method for determining antimicrobial minimum inhibition concentrations (MICs) was utilised for screening of crude extracts and for bioactivity-guided isolation of antimicrobial compounds.[16] Antimicrobial studies on the South African Combretaceae species also involved the use of thin-layer chromatography (TLC) for the identification of suitable extractants and to estimate the number of antimicrobial compounds present in the crude extract.[7,17] Bioautography is the use of TLC to determine the biological activities of individual components in a crude extract, following their separation using a suitable mobile phase. It can be applied to reveal compounds with antimicrobial activity towards a specific bacterial or fungal pathogen.[7,18,19] Unlike the MIC assay that reflects the combined activities of the components, bioautography highlights the activity of individual active compounds.[20] The technique also provides easy identification of compounds with activity that may be lost during MIC-guided fractionation, particularly where the active compound is present at a low concentration within the plant extract.

Although TLC or high-performance thin-layer chromatography (HPTLC) is used to identify individual antimicrobial compounds in plant species, the coupling to mass spectrometry (MS) offers a useful tool for the identification of bands that were revealed as having activity through bioautography. The HPTLC–MS technique involves the direct identification of active compounds by extracting bands corresponding to activity, from an identical HPTLC reference plate, through the use of a TLC interface. The eluent is infused directly into the MS, where it is analysed and a spectrum is obtained.[21] This high throughput technique provides insights into compounds that could easily be overlooked during the process of classical bioactivity-guided isolation. Studies have reported the application of HPTLC–MS to phytochemical, anti-oxidant, anticholinesterase and antimicrobial studies, as well as to quality control.[19,22–24] However, there are no reports on the application of HPTLC–MS to identify antimicrobial compounds in Combretaceae species.

Besides the screening of, and isolation from individual species, studies related to the isolation of antimicrobial compounds from taxonomically-related species within Combretaceae have been reported. While some of these were comparative studies, others were carried out with the aim of selecting suitable species for further investigation of their active constituents. Six South African *Terminalia* species were evaluated for their antifungal activities against common pathogens related to veterinary use, and *T. sericea* and *T. brachysemma* were identified as the most promising, as judged from the number of inhibition zones revealed through bioautography.[20] In a subsequent study, 24 *Combretum* species were evaluated for their antifungal activities towards *Candida albicans*, *Cryptococcus neoformans*, *Aspergillus fumigatus*, *Microsporum canis* and *Sporothrix schenckii* using a bioautography approach.[25] The results indicated that species belonging to the section *Hypocrateropsis* Engl. & Diels (*Combretum celastroides* subsp. *celastroides*, *Combretum celastroides* subsp. *orientale*, *Combretum imberbe*, *Combretum padoides*) displayed the largest number of inhibition zones associated with antimicrobial compounds, but no attempt was made to identify the compounds. The antimicrobial activities of six *Combretum* (*C. collinum*, *C. erythrophyllum*, *C. hereroense*, *C. microphyllum*, *C. molle*) and two *Terminalia* (*T. pruinoides*, *T. sericea*) species towards 19 micro-organisms, including bacteria, fungi and a yeast, determined using the microdilution assay, indicated that the *Terminalia* species were more active than the *Combretum* species.[26]

Although Combretaceae species are reported to be active against microbial pathogens, the process of identifying active compounds is tedious. It involves testing of the crude extract for activity, followed by bioactivity-guided fractionation and eventually the purification of a few active compounds. In the case of Combretaceae, similarities in the chemistry and antimicrobial activities make it challenging to easily identify suitable species for commercial purposes.[1,27] It is laborious to purify crude extracts from individual species and discouraging to identify the same compounds from several plant species. To date, the use of bioautography for high throughput screening of Combretaceae species has not produced the desired result. The most recent bioautography study[28] involved exploring antifungal and antibacterial compounds in fractions of *Combretum molle*. However, the study was limited to reporting of the MIC values and the inhibition zones for each fraction. Further identification of the active compounds was not accomplished. In a recent study,[29] we correlated the chemistry of 51 methanolic extracts of Combretaceae (35 species) to their antimicrobial activities determined using the microdilution assay towards nine pathogenic

bacteria. Biochemometric analyses enabled the prediction of active compounds towards specific bacteria. In this follow-up study, we aimed to further investigate compounds contributing to the antimicrobial activities of the Combretaceae species as revealed through bioautography, followed by HPTLC–MS to identify active compounds and confirm the biochemometric predictions.

## 2 | EXPERIMENTAL

### 2.1 | Sample and pathogen selection for bioautography

Seventy-seven samples, representing 35 species from four genera (*Combretum*, *Pteleopsis*, *Quisqualis*, *Terminalia*) of Combretaceae (Supporting Information Table S1) were collected from Lowveld National Botanical Garden (Coordinate: 22°42′12.384S 30°34′27.702E) in Mbombela, Buffelskloof Private Nature Reserve (25°19′25.0S 30°29′15.4E) and Thengwe in Venda (22°46'52S 30°32′36E). Samples from Lowveld National Botanical Garden were identified by Dr Willem Froneman while samples from Buffelskloof Private Nature Reserve were authenticated by Mr. John Burrows. Voucher specimens for both localities were prepared and deposited in the Department of Pharmaceutical Sciences, Tshwane University of Technology (Pretoria, South Africa). Samples from Thengwe were identified by Dr Masevhe and voucher specimen deposited in the Department of Botany, University of Venda (Thohoyandou, South Africa). The methanol extracts were analysed using ultra-performance liquid chromatography coupled with mass spectrometry (UPLC–MS). Chemometric analysis of the aligned data, comprising retention time_mass (Rt_*m/z*) pairs, together with the corresponding peak areas (*X*-variables), was carried out using metaboanalyst 5.0 (Xia Lab, McGill University, Montreal, Canada), an open source metabolomics software (www.metaboanalyst.ca/MetaboAnalyst/home.xhtml).[30] The UPLC–MS data obtained were used for hierarchical cluster analysis (HCA). A total of 15 samples of species representing the chemical diversity within the family were selected from the three branches (X, Y or Z), after observing the clustering patterns on the dendrogram. Species for which noteworthy MIC values had been previously recorded[29] were also included. Of the nine bacterial pathogens used in the previous study, four test organisms (two Gram-positive and two Gram-negative) demonstrating the highest susceptibility, were selected for compound identification (Table 1).

**TABLE 1** List of 15 plants from the genera *Combretum* and *Terminalia* selected from the dendrogram (Figure 1) obtained from hierarchical cluster analysis (HCA) of ultra-performance liquid chromatography–mass spectrometry (UPLC–MS) data. The minimum inhibition concentration (MIC) values (mg/mL) previously obtained,[29] are also listed

| Sample number | Species[a] | *Staphylococcus aureus* (ATCC 25923) | *Bacillus cereus* (ATCC 11778) | *Escherichia coli* (ATCC 8739) | *Salmonella typhimurium* (ATCC 14028) |
|---|---|---|---|---|---|
| **Branch X** | | | | | |
| 1 | *Combretum kraussii* Hochst. | 1.00 | 0.25 | 1.50 | 0.50 |
| 2 | *Combretum woodii* Dummer | 1.50 | 1.50 | 1.25 | 0.50 |
| **Branch Y** | | | | | |
| 3 | *Combretum zeyheri* Sond. | 2.00 | 1.25 | 1.50 | 0.38 |
| **Branch Z** | | | | | |
| 4 | *Combretum acutifolium* Exell. | 0.38 | 0.15 | 0.63 | 0.98 |
| 5 | *Combretum apiculatum* Sond. | 3.00 | 0.25 | 0.75 | 0.50 |
| 6 | *Combretum bracteosum* (Hochst.) Engl. & Diels | 2.00 | 0.50 | 2.00 | 1.03 |
| 7 | *Combretum collinum* subsp. *suluense* (Engl. & Diels) Okafa | >3.00 | 0.63 | 1.50 | 0.75 |
| 8 | *Combretum elaeagnoides* Klotzsch | 0.15 | 0.28 | 0.75 | 0.50 |
| 9 | *Combretum hereroense* Schinz | 2.00 | 0.50 | 1.25 | 0.50 |
| 10 | *Combretum imberbe* Wawra | 0.09 | 0.27 | 0.63 | 0.50 |
| 11 | *Combretum microphyllum* Klotzsch | 2.50 | 0.50 | 1.00 | 0.75 |
| 12 | *Combretum molle* R.Br. ex G.Don | 2.50 | 0.32 | 1.75 | 0.75 |
| 13 | *Combretum padoides* Engl. & Diels | 1.50 | 0.16 | 1.25 | 0.25 |
| 14 | *Terminalia sambesiaca* Engl. & Diels | 2.00 | 0.38 | 1.50 | 0.38 |
| 15 | *Terminalia sericea* Burch. ex DC. | >3.00 | 0.64 | 1.25 | 0.63 |
| | Ciprofloxacin control (μg/mL) | 0.08 | 0.04 | 1.25 | 0.04 |

[a]The Plant List (http://www.theplantlist.org/) was used to verify plant names.

## 2.2 | Sample preparation for bioautography

Each powdered sample (10.0 g) was sonicated (LIBM8, Labcon, South Africa) in 100 mL of methanol (AR grade; Thembane Chemicals, Johannesburg, South Africa) for 30 min. After allowing the mixture to stand overnight, it was filtered (Whatman No 1). The solid material was mixed with a second 100 mL aliquot of methanol for 15 min, and the mixture was subsequently sonicated for 30 min. After filtering, the two filtrates were combined and concentrated to dryness using a centrifugal evaporator (Genevac EZ 2 plus; Ipswich, UK), and the mass of the residue determined by difference. The dried methanol extracts of the 15 species (Table 1), selected from the dendrogram, were redissolved in appropriate volumes of methanol to prepare solutions with a final concentration of 20 mg/mL for bioautography analysis. The solutions were filtered through 0.45 μm nylon syringe filters (Acrosdisc®; Pall, New York, NY, USA) into individual vials for analysis.

## 2.3 | High-performance thin-layer chromatography (HPTLC) analysis

A CAMAG semi-automated system with VisionCat version 2.5 planar chromatography manager software (Camag, Muttenz, Switzerland) was used for HPTLC analysis of the sample extracts. The system comprises an automated TLC Sampler 4, TLC visualiser, automatic developing chamber ADC2, Camag derivatiser and TLC Plate Heater III. Methanol extracts were applied to the HPTLC silica gel 60 $F_{254}$ (20 cm × 10 cm) plates (Merck, Johannesburg, South Africa) as 8 mm bands. The plates were developed after optimising the mobile phase [ethyl acetate:formic acid:water (50:3:3)] for best resolution of the sample extracts. The chamber was saturated for 20 min at 33% relative humidity and 23 ± 2 °C, using 25 mL of the solvent. Prepared plates were developed by allowing the solvent (10 mL) to migrate to a distance of 70 mm from the plate edge. Once the developing solvent was optimised, three sets of plates were prepared. The first set was used for bioautography analysis to test against each of the four pathogens. The second was derivatised with 10% methanolic sulphuric acid to visualise the zones of inhibition, while plates in the third set were developed and later used to mark areas corresponding to inhibition zones on the plates for HPTLC–MS analysis.

## 2.4 | Bioautography analysis

Cultures of *Staphylococcus aureus* (ATCC 25923), *Bacillus cereus* (ATCC 11778), *Escherichia coli* (ATCC 8739), and *Salmonella typhimurium* (ATCC 14028) [Davies Diagnostics (Pty) Ltd, Randburg, South Africa] were individually prepared in Oxoid Tryptone Soya broth (TSB) [Quantum Biotechnologies (Pty) Ltd, Johannesburg, South Africa] by incubation (EcoTherm, Hartkirchen, Austria) at 37 °C for 24 h. These were diluted with TSB to prepare an inoculum with a concentration of approximately $1 \times 10^6$ colony forming unit (CFU)/mL. The McFarland turbidity standard (0.5) solution was used to estimate the concentration through visual inspection.

Bioautography assays were carried out on the methanol extracts of the 15 selected plant species (Table 1) as described by Van Vuuren *et al.*[31] The extracts (10 μL) were applied as bands (8 mm wide) to the silica gel HPTLC plates (10 cm × 20 cm) at a concentration of 20 mg/mL. Four identical plates were prepared for each of the pathogens tested. A sterile bioautography glass box was filled with 150 mL Oxoid Tryptone Soya agar [TSA; Quantum Biotechnologies (Pty) Ltd] and allowed to solidify. Each developed HPTLC plate was sterilised under ultraviolet (UV) light for 1 h and subsequently placed face-up on the surface of the solid base. It was then overlaid with TSA (200 mL) containing the appropriate bacterial culture ($1 \times 10^6$ CFU/mL). The plates were incubated for 24 h at 37 °C. The inhibition zones, characterised by cream-white areas against a pink background, were visualised 24 h after spraying the surface of the agar with *p*-iodonitrotetrazolium violet (INT; Sigma-Aldrich, St Louis, MO, USA). One of the two reference HPTLC plates was sprayed with 10% methanolic sulphuric acid and heated, for visualisation of the bands. Active zones corresponding to the bioautography zones of bacterial inhibition were marked with a pencil on the remaining plate (consisting of separated extracts only) after comparing the retardation factors (Rfs) with the visualised bands, and this plate was used for the HPTLC–MS analysis.

## 2.5 | High-performance thin-layer chromatography–mass spectroscopy (HPTLC–MS) analysis

The CAMAG HPTLC–MS interface (oval elution head, 4 mm × 2 mm; Muttenz, Switzerland) was used to elute compounds from the marked bands. The inlet of the flow pump of the instrument was connected to a UPLC pump (Waters Corp., Milford, MA, USA) and the outlet to a quadrupole Time-of-Flight (qToF) mass spectrometer (Waters Corp., Milford, MA, USA). Acetonitrile (UPLC grade, Romil Ltd, Cambridge, UK), at a flow rate of 0.1 mL/min, was used as an eluent to remove the target compounds from the silica gel plate (running time: 1 min). Following electrospray ionisation (ESI) of the eluate, mass spectra were acquired in the negative mode. Conditions for the $ESI^-$ were: capillary voltage, 2500 V; sampling cone, 40 V; source temperature, 100 °C; desolvation temperature, 400 °C. Nitrogen served as the desolvation gas at a flow rate of 600 L/h. MassLynx 4.1 software (Waters Corp., Milford, MA, USA) was used for instrument control and data acquisition.

## 2.6 | HPTLC analysis to investigate the presence of antibacterial compounds in Combretaceae

A second sample extraction was carried out to investigate chemotaxonomic variation of the antimicrobial compounds within Combretaceae. This part of the study, conducted to visually estimate the presence of compounds revealed to have antibacterial activity, encompassed 35 species (number of samples = 39). The species included in the

investigation are: *Combretum* [*C. acutifolium* Exell, *C. adenogonium* Steud. ex A.Rich., *C. albopunctatum* Suess., *C. apiculatum* Sond., *C. bracteosum* (Hochst.) Engl. & Diels, *C. caffrum* (Eckl. & Zeyh.) Kuntze, *C. celestroides* subsp. *celestroides* Welw. ex M.A.Lawson, *C. celestroides* subsp. *orientale* Exell, *C. collinum* subsp. *suluense* (Engl. & Diels) Okafa, *C. edwardsii* Exell, *C. elaeagnoides* Klotzsch, *C. erythrophyllum* (Burch.) Sond., *C. hereroense* Schinz, *C. imberbe* Wawra, *C. kraussii* Hochst., *C. microphyllum* Klotzsch, *C. mkuzense* J.D. Carr & Retief, *C. molle* R.Br. ex G. Don, *C. nelsonii* Dummer, *C. oxystachyum* Welw. ex M.A.Lawson, *C. padoides* Engl. & Diels, *C. paniculatum* Vent., *C. petrophilum* Retief, *C. woodii* Dummer, *C. vendae* A.E.van Wyk, *C. zeyheri* Sond.], *Terminalia* (*T. brachystemma* subsp. *brachystemma* Welw. ex Hiern, *T. gazensis* Baker f., *T. mollis* M.A.Lawson, *T. phanerophlebia* Engl. & Diels, *T. prunioides* M.A.Lawson, *T. sambesiaca* Engl. & Diels, *T. sericea* Burch. ex DC.), *Pteleopsis* (*P. myrtifolia* M.A.Lawson), *Quisqualis* (*Q. littorea* Engl.)

Each powdered sample (1.0 g) was sonicated in 10 mL methanol for 30 min. The mixture was filtered through a 0.45 μm nylon syringe filter and transferred to individual vials. Samples were applied as 8 mm bands by applying 2 μL aliquots. The plates were developed as described, where after either 10% sulphuric acid in methanol for the detection of triterpenoids, or Natural Product Reagent for the detection of flavonoids,[32] was applied. To detect triterpenoids, the plates were heated for 3 min after application of the reagent, until the bands were visible.

## 3 | RESULTS AND DISCUSSION

### 3.1 | Selection of bacterial pathogens and samples

In our previous study, the susceptibility of nine bacterial species, related to conditions of the gastrointestinal tract and wound infections, were used to evaluate the antibacterial activities of the methanol extracts representing 35 species of Combretaceae.[29] The microdilution assay was used to determine the MIC values of 51 extracts. Four of the nine pathogens were selected for this in-depth follow-up study of the antimicrobial activity of Combretaceae species, namely *B. cereus* and *Salmonella typhimurium*, which were the most susceptible of the pathogens, and *S. aureus* and *E. coli*, because the extracts displayed variable activity towards these pathogens. A biochemometric analysis, carried out as part of the previous study by combining the MIC values and the UPLC–MS data, indicated that the triterpenoids contributed to the activity of the species. In this study, rather than applying bioautography to all 35 species collected, two criteria were used to select 15 samples considered to be representative of the family, for identification of compounds with antimicrobial activity. The most important criterion was the chemistry of the species, which was interpreted through HCA of the UPLC–MS data, previously obtained through analysis of the methanol extracts of all 77 samples, as described.[29] The dendrogram (Figure 1) obtained after further analysis of the same data indicates three distinct clusters (X, Y and Z), reflecting three groupings based on the chemical profiles, with further sub-branches reflecting chemical variation within samples in a cluster. Samples of the same species clustered tightly. Fifteen representative samples (Figure 1; Table 1) were selected from the three branches and from most of the sub-branches, to represent the chemical diversity within the family.

### 3.2 | Bioautography analysis and band correlation

The bioautography approach provides insight into the contribution of individual components to the overall activity of an extract.[20] This technique indicates whether a major compound with strong

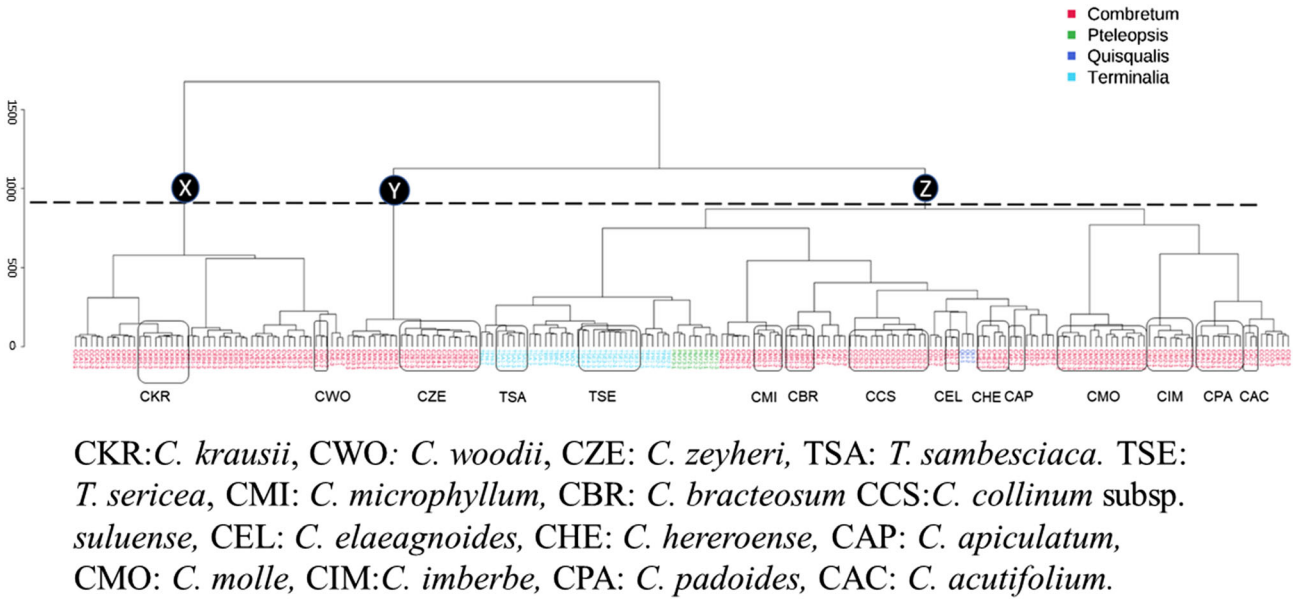


**FIGURE 1** Dendrogram derived from hierarchical cluster analysis of the UPLC–MS data obtained through analysis of 77 samples of Combretaceae. The blocks indicate the 15 species selected for bioautography.

antimicrobial activity is present, or whether the additive effect of several minor compounds contribute to the antimicrobial activity of an extract. We also explored if the same compounds present in different species were active towards the pathogens, and whether some compounds are more active than others towards a specific pathogen. The bioautographic plates for selected species displaying inhibition zones are presented in Figure 2. Seven species (*Combretum padoides*, *Combretum zeyheri*, *Combretum bracteosum*, *Combretum microphyllum*, *Combretum imberbe*, *Combretum molle*, *Combretum elaeagnoides*) displayed inhibition zones against *B. cereus* (Figure 2A). Similarly, seven species (*Combretum krausii*, *Combretum woodii*, *Combretum acutifolium*, *Combretum apiculatum*, *Combretum imberbe*, *Combretum elaeagnoides* *Combretum hereroense*) displayed inhibition of *S. aureus* (Figure 2B). Compounds present in the extracts of seven species (*Combretum krausii*, *Combretum woodii*, *Combretum acutifolium*, *Combretum padoides*, *Combretum bracteosum*, *Combretum imberbe*, *Combretum elaeagnoides*) inhibited *E. coli* (Figure 2C). Five species (*Combretum krausii*, *Combretum zeyheri*, *Combretum bracteosum*, *Combretum imberbe*, *Combretum elaeagnoides*) displayed inhibition zones against *Salmonella typhimurium* (Figure 2D). Only two of the 15 selected species (*Combretum imberbe*; Figure 2, track 12) and *Combretum elaeagnoides* (Figure 2, track 14) displayed inhibition zones across all four pathogens, indicating potential broad-spectrum activity. While several inhibition zones were evident in the extract of *Combretum imberbe*, one broad inhibition zone was present in *Combretum elaeagnoides*; this zone was particularly prominent when the extract was tested against *S. aureus* and *E. coli*. In our previous study, *Combretum imberbe*, *Combretum elaeagnoides* and *Combretum acutifolium* were the most active against all the pathogens with MIC values < 1.0 mg/mL (Table 1). *Combretum imberbe* (Figure 2, track 12) displayed the most inhibition zones against the four pathogens. This finding is consistent with the results of our previous study, which indicated that the species was the most active of those tested, as reflected by the lowest average MIC of 0.40 mg/mL.[29]

The next step was to identify bands (Rf values) corresponding to the zones of inhibition observed on the bioautography plates.

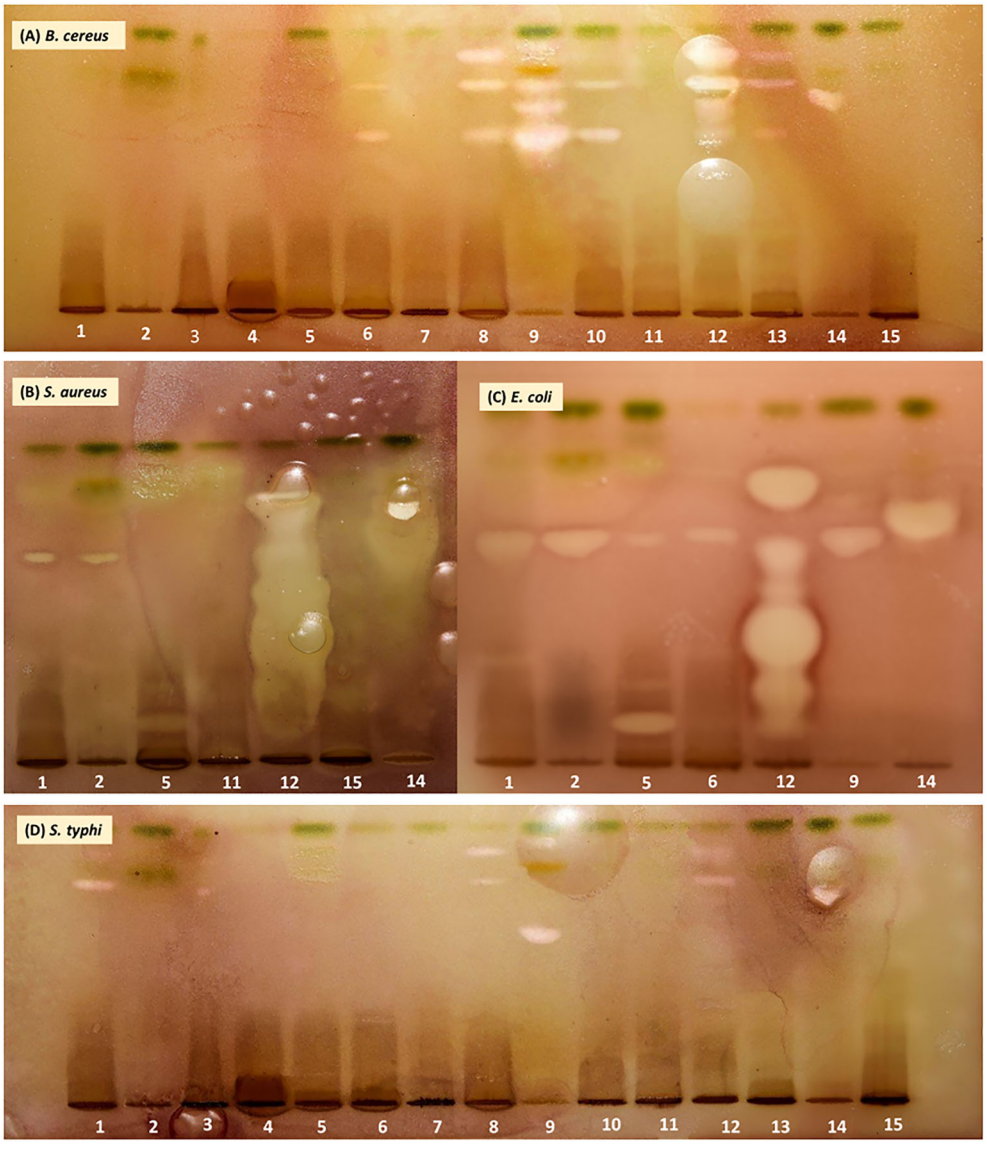


**FIGURE 2** Bioautography plates for the analysis of 15 Combetaceae species against (a) *Bacillus cereus*, (B) *Staphylococcus aureus*, (C) *Escherichia coli*, (D) *Salmonella typhimurium*. Track numbers: (1) *Combretum krausii*, (2) *Combretum woodii*, (3) *Terminalia sericea*, (4) *Terminalia sambesciaca*, 5) *Combretum acutifolium*, (6) *Combretum padoides*, (7) *Combretum collinum* subsp. *suluense*, (8) *Combretum zeyheri*, (9) *Combretum bracteosum*, (10) *Combretum microphyllum*, (11) *Combretum apiculatum*, (12) *Combretum imberbe*, (13) *Combretum molle*, (14) *Combretum elaeagnoides*, (15) *Combretum hereroense*.

Fingerprints of the 15 methanol extracts were obtained and compared to the bioautography plates, following derivatisation with sulphuric acid. Inhibition zones on the bioautography plates were located between Rf values 0.5 to 0.9 (Figure 2A). The compounds corresponding to these bands are characteristic of triterpenoids (Figure 3A), while compounds corresponding to the inhibition zones between 0.09 and 0.25 reflect the presence of flavonoids (Figure 3B).[32]

The inhibition zones corresponding to Rf 0.57 were active against the four pathogens. However, species were selective in the pathogens inhibited. For example, with *Combretum krausii* (Figure 2B,C, track 1) and *Combretum woodii* (Figure 2B, C, track 2), the compound (Figure 3A, tracks 1 and 2; blue rectangle) inhibited *S. aureus* and *E. coli*, whereas the compound with Rf 0.57 inhibited only *E. coli* (Figure 3A, track 5; green rectangle) in the extract of *Combretum acutifolium* (Figure 1C, track 5). The compound inhibited only *B. cereus* and *E. coli* (Figure 3A, tracks 6 and 12; red rectangle) when present in *Combretum padoides* (Figure 2A,C, track 6) and *Combretum imberbe* (Figure 2A,C, track 12), while *B. cereus* only (yellow rectangle) was inhibited at Rf 0.57 by *Combretum zeyheri* (Figure 2A, track 8), *Combretum microphyllum* (Figure 2A, track 10) and *Combretum molle* (Figure 2A, track 13). The compound also inhibited *B. cereus*, *E. coli* and *Salmonella typhi* (Figure 3A, track 9; light green rectangle) in the extract of *Combretum bracteosum* (Figure 2A,C,D, track 9). A similar trend was observed for the compound corresponding to Rf 0.71. With *Combretum kraussii*, only *Salmonella typhimurium* was inhibited (Figure 2D, track 1; Figure 3A, track 1; gold rectangle), while *B. cereus* and *E. coli* were inhibited by *Combretum padoides* (Figure 2A, track 6; Figure 2C, track 4; Figure 3A, track 6; red rectangle). Again, *B. cereus* and *Salmonella typhimurium* were inhibited by the same compound present in *Combretum zeyheri* (Figure 2D, track 8; Figure 3A, track 8; light blue). With *Combretum imberbe*, all the pathogens (black rectangle) were inhibited by the compound at Rf 0.71 (Figure 2A–D, track 12). The compounds in the extract of *Combretum padoides* (Rf 0.57, 0.71) inhibited *E. coli* only. The compound corresponding to Rf 0.77 inhibited the same pathogens (*S. aureus* and *E. coli*) in the extracts of *Combretum kraussii*, *Combretum woodii* and *Combretum acutifolium*.

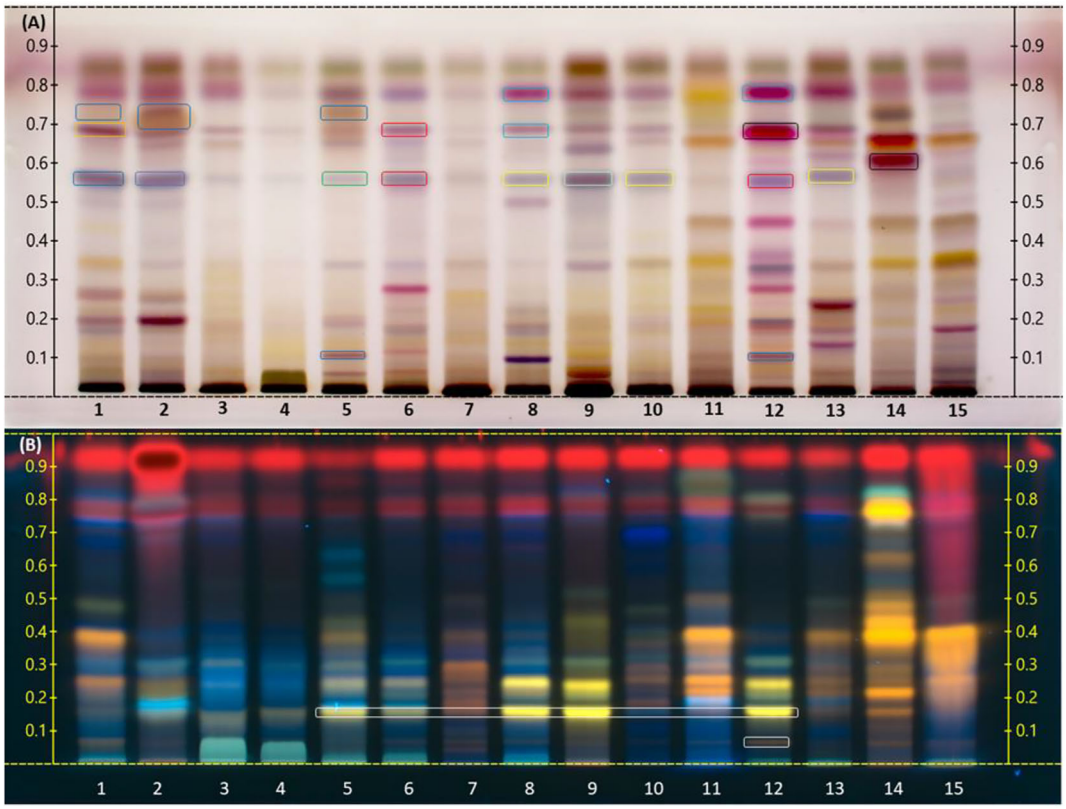


**FIGURE 3** HPTLC analysis of samples representing the 15 species from the family Combretaceae. Track numbers: (1) *Combretum krausii*, (2) *Combretum woodii*, (3) *Terminalia sericea*, (4) *Terminalia sambesciaca*, (5) *Combretum acutifolium*, (6) *Combretum padoides*, (7) *Combretum collinum* subsp. *suluense*, (8) *Combretum zeyheri*, (9) *Combretum bracteosum*, (10) *Combretum microphyllum*, (11) *Combretum apiculatum*, (12) *Combretum imberbe*, (13) *Combretum molle*, (14) *Combretum* elaeagnoides, (15) *Combretum hereroense.* (A) Derivatised with 10% sulphuric acid in methanol and visualised under white light. The blue rectangles indicate activity towards *Staphylococcus aureus* and *Escherichia coli*, the light green rectangle indicates activity towards *Bacillus cereus*, *E. coli* and *Salmonella typhimurium*, the red rectangles indicate activity towards *B. cereus* and *E. coli*, while the light blue boxes indicate activity towards *B. cereus* and *Salmonella typhimurium*. The following rectangles indicate activity towards only one pathogen, namely *B. cereus* (yellow), *E. coli* (green), *Salmonella typhimurium* (gold) and the black rectangle represent zones active against all pathogens. (B) Derivatised with Natural Product Reagent and viewed under UV light (366 nm radiation). The white bar indicates the presence of flavonoids.

This compound is not a triterpenoid, as deduced from the colour of the bands. *Combretum elaeagnoides* did not display a band at Rf 0.71. However, the compound corresponding to Rf 0.60 inhibited all four pathogens (black rectangle). The compound corresponding to Rf 0.84 in extracts of *Combretum zeyheri* and *Combretum imberbe*, inhibited *B. cereus* and *Salmonella typhimurium* (light blue rectangle). The compound corresponding to Rf 0.09 in *Combretum acutifolium* and *Combretum imberbe* inhibited *S. aureus* and *E. coli* (blue rectangle).

The flavonoid corresponding to the rectangle at Rf 1.7 (Figure 3B) was present in *Combretum acutifolium* (track 5), *Combretum zeyheri* (track 8), *Combretum bracteosum* (track 9), and *Combretum imberbe* (track 12), However, only *Combretum acutifolium* and *Combretum imberbe* exhibited inhibition zones when tested against *S. aureus* (Figure 1B, tracks 3 and 5) and *E. coli* (Figure 1C, tracks 3 and 5). It is possible that the compounds in *Combretum zeyheri* and *Combretum bracteosum* are different from the flavonoids that occur in the other two. The highest intensity of the bands corresponding to Rf 0.57 was visible in extracts of *Combretum krausii* (track 1), *Combretum woodii* (track 2), *Combretum padiodes* (track 6), *Combretum bractesum* (track 9) and *Combretum microphyllum* (track 10). Of the five species, only *Combretum microhyllum* did not display an inhibition zone against *E. coli*. *Combretum acutifolium* (track 5), which had low intensity of the band, did not display inhibition. However, *Combretum padiodes*, *Combretum zeyheri*, *Combretum bracteosum*, *Combretum microphyllum*, *Combretum imberbe* and *Combretum molle* displayed inhibition zones at Rf 0.57 towards *B. cereus* (Figure 2A). Collectively, all the species with a band visible at Rf 0.57 displayed inhibition against *B. cereus* or *E. coli*, or both pathogens. Against *S. aureus* (Figure 3B), only *Combretum kraussii* and *Combretum woodii* displayed an inhibition zone at Rf 0.57. Strong overlapping of the inhibition bands of *Combretum imberbe* when tested against *S. aureus* and *E. coli* suggests additive effects of the compounds. This trend was not observed against *B. cereus* and *Salmonella typhimurium*. Unlike the activity against the other three pathogens, *Combretum imberbe* displayed only two faint inhibition

**TABLE 2** Active compounds identified from the molecular ion and fragments ions using high-performance thin-layer chromatography–mass spectrometry (HPTLC–MS).

| Retardation factor (Rf) value | *m/z* $[M - H]^-$ (fragment ions) | Class/name | Species | Pathogen inhibited |
|---|---|---|---|---|
| 0.09 | 447 (431, 357, 301) | Flavonoid | *Combretum imberbe*, *Combretum acutifolium* | *Staphylococcus aureus*, *Escherichia coli* |
| 0.17 | 447 (431, 357, 301) | Flavonoid | *Combretum imberbe*, *Combretum acutifolium* | *Staphylococcus aureus*, *Escherichia coli* |
| 0.25 | 495 (333) | Combretastatin B1 glycoside | *Combretum krausii* | *Escherichia coli* |
| 0.25 | 431 (311) | Flavonoid | *Combretum imberbe* | *Staphylococcus aureus*, *Escherichia coli* |
| 0.33 | 757 (471) | Imberbic acid glycoside derivative | *Combretum imberbe* | *Staphylococcus aureus*, *Escherichia coli* |
| 0.41 | 753 (471) | Imberbic acid glycoside derivative | *Combretum imberbe* | *Staphylococcus aureus*, *Escherichia coli* |
| 0.50 | 755 (471) | Imberbic acid glycoside derivative | *Combretum imberbe* | *Staphylococcus aureus*, *Escherichia coli* |
| 0.57 | 487 | Arjunic acid | *Combretum krausii*, *Combretum woodii*, *Combretum acutifolium*, *Combretum padoides*, *Combretum bractesum* | *Staphylococcus aureus*, *Bacillus cereus*, *Escherichia coli Salmonella typhimurium* |
| 0.60 | 499 | Jessic acid | *Combretum elaeagnoides* | *Staphylococcus aureus*, *Bacillus cereus*, *Escherichia coli Salmonella typhimurium* |
| 0.66 | 289 | Epicatechin/catechin | *Combretum elaeagnoides* | *Staphylococcus aureus* |
| 0.69 | 471/943 | Imberbic acid | *Combretum imberbe* | *Staphylococcus aureus*, *Bacillus cereus*, *Escherichia coli Salmonella typhimurium* |
| 0.71 | 471 | Triterpenoid | *Combretum krausii*, *Combretum imberbe*, *Combretum zeyheri* | *Bacillus cereus*, *Salmonella typhimurium* |
| 0.77 | 319 | Combretastatin B5 | *Combretum woodii* | *Staphylococcus aureus*, *Escherichia coli* |
| 0.85 | 455 | Ursolic acid | *Combretum bractesum*, *Combretum imberbe*, *Combretum molle* | *Bacillus cereus*, *Salmonella typhimurium* |
| 0.84 | 255 | Pinocembrin | *Combretum apiculatum* | *Staphylococcus aureus* |

zones towards *Salmonella typhimurium*. Although *Salmonella typhimurium* was one of the most susceptible pathogens in the MIC assay (Table 1), only a few inhibition zones were revealed, suggesting that the individual compounds may be highly active (Figure 3D). No inhibition zones were observed for the extracts of *T. sericea*, *T. sambesciaca* or *Combretum collinium*. The antibacterial compound at Rf 0.57 was present in all three samples, but at relatively low concentrations, as indicated by the weak intensities of the bands.

## 3.3 | Identification of antibacterial compounds

After the correlation of the bioautography plates with the derivatised HPTLC plates, the compounds corresponding to the inhibition zones were identified using HPTLC–MS. Mass fragments were compared to values reported in the literature, or available standards were used to identify the compounds. The molecular ion *m/z* 447, corresponding to Rf 0.09 and 0.17 (Table 2), was correlated to flavonoids, due to the characteristic yellow bands on the HPTLC plate (Figure 3B). The molecular ion $[M - H]^-$ at *m/z* 431 (Rf 0.25) produced the fragment ion *m/z* 311, indicating the presence of vitexin, suggesting the presence of a flavonoid.[33] Although both *Combretum kraussii* and *Combretum imberbe* displayed compounds at the same Rf (0.17) value, the colours of the bands (Figure 3B) indicate that they are different compounds. The molecular ion $[M - H]^-$ at *m/z* 447 (Rf 0.09, 0.17) produced fragment ions *m/z* 431, 357, 301 which indicates the presence of luteolin glycosides.[33] The molecular ion $[M - H]^-$ at *m/z* 495 (Rf 0.25) displayed a fragment ion at *m/z* 333, indicating the loss of a sugar moiety (162 amu). The fragmentation pattern corresponds to reported values for combretastatin B1 glycoside.[34] The molecular ions *m/z* 757, 753 and 755 all displayed a fragment of *m/z* 471, indicating the presence of imberbic acid derivatives. The molecular ion at *m/z* 487 corresponds to reported values for arjunic acid, which is a known antibacterial agent.[10] It was identified as a major antibacterial compound against *E. coli* in this study. The molecular ion at *m/z* 499 was identified as jessic acid, which was previously isolated from *Combretum elaeagnoides*.[35] It inhibited all four pathogens, and it is the major antibacterial constituent of *Combretum elaeagnoides*. The molecular ion at *m/z* 289 was identified as catechin/epicatechin and displayed possible additive activity with jessic acid against *S. aureus* as indicated by the broad band (Figure 2B, track 14). The molecular ion *m/z* 471 was identified as imberbic acid and was previously reported as a prominent antibacterial compound in the *Combretum imberbic* extract.[36,37] The molecular ion *m/z* 319 was identified as combretastatin B5 and inhibited *E. coli* (Figure 2C, track 2). Combretastatin B5 was identified as the major antibacterial compound of *Combretum woodii*.[7] This compound was not detected in any other sample. The molecular ion at *m/z* 255 (Rf 0.84) was identified as pinocembrin. It was previously isolated and identified from *Combretum apiculatum*,[8] and inhibited the growth of *S. aureus* (Figure 2B, track 11). The band corresponding to the Rf 0.85 was identified as ursolic acid (*m/z* 455) using a reference standard.

## 3.4 | Investigation of the chemotaxonomic significance of antibacterial compounds

The presence of the identified major antibacterial compounds was explored in 39 samples, representing 35 species within Combretaceae. Unlike the extracts used for bioautography (Figures 2 and 3), the concentration of the extracts used for the chemotaxonomy study were not artificially adjusted to a fixed concentration, but were prepared to reflect the actual concentration of each constituent in the extract. Visual observation of the concentration (intensity of the bands) of the compounds was used to predict the abundance, which was then correlated with the inhibition observed on the bioautographic plates (Figure 4). *Combretum* species belonging to the section *Angustimarginata* (Figure 4, tracks A1–A6) contained high concentrations of arjunic acid (Rf 0.57), with the exception of *Combretum erythrophyllum*. In this study it was established that arjunic acid (or possibly an isomer of arjunic acid) is very effective against *E. coli*. Arjunic acid isolated from *T. sericea* root displayed antibacterial activities towards *E. coli* with a reported MIC value of 3.5 μg/mL which was comparable to the positive control neomycin (MIC 3.0 μg/mL).[10] The extract of *Combretum molle* (Figure 4, track A7), which belongs to the section *Ciliatipetala* Engl. & Diels., was also rich in arjunic acid. An inhibition zone corresponding to arjunic acid was observed in the extract of *Combretum molle* against *B. cereus*. Other species in the same section (Figure 4, tracks A8–A11) contained only low concentrations of the compound. Inspection of the plates indicates that arjunic acid (or possibly an isomer of the compound) is present in almost all the samples and is therefore ubiquitous in the Combretaceae species. This compound may have been responsible for the activity of the samples, as reflected by MIC values obtained in our previous study.[29] Only five species (*Combretum vendae* – Figure 4, track A4; *Combretum molle* – Figure 4, track A7; *Combretum imberbe* – Figure 4, track B4; *Combretum zeyheri* – Figure 4, track B5; *Combretum mkuzense* – Figure 4, track B6) displayed intense bands corresponding to ursolic acid (Rf 0.85). Against *B. cereus*, distinct inhibition zones were evident for *Combretum zeyheri* and *Combretum imberbe* at Rf 0.85, indicating that ursolic acid was responsible for the activity. Similarly, *Combretum zeyheri* and *Combretum imberbe* displayed inhibition against *Salmonella typhi*. This indicates that ursolic acid is active against both *B. cereus* and *Salmonella typhi* and that the differences in the activity of the species are related to its variable concentration in the extracts. Studies have reported the antibacterial activity of ursolic acid,[36] however, there is no available literature on the antibacterial activity of ursolic acid from Combretaceae species. Since ursolic acid is present in all the samples used in this study, it implies that the compound contributed to the general antibacterial activity of these species. In our previous study,[29] *B. cereus* (MIC 0.52 mg/mL) was the most susceptible pathogen, followed by *Salmonella typhimurium* (MIC 0.63 mg/mL). Species belonging to section *Angustimarginata* have a high arjunic acid content and would most likely be active against *S. aureus* and *E. coli*, provided there are no antagonistic compounds present in the extract matrix.

The use of bioautography further validates our hypothesis that triterpenoids contribute to the antibacterial activities of South African

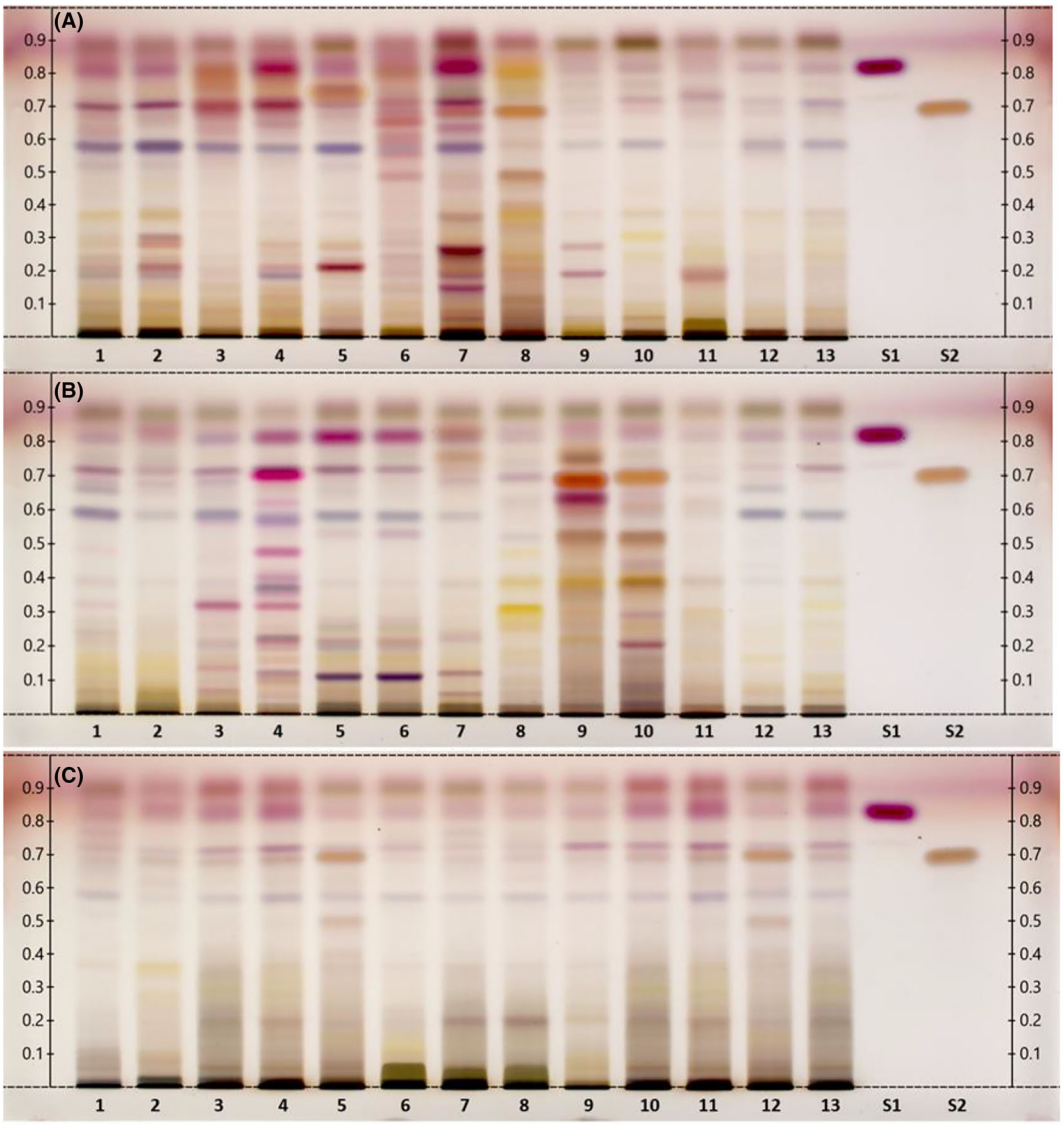


**FIGURE 4** HPTLC profiles of (A) *Combretum caffrum* (A1), *Combretum kraussii* (A2), *Combretum nelsonii* (A3), *Combretum vendae* (A4), *Combretum woodii* (A5), *Combretum erythrophylum* (A6), *Combretum molle* (A7), *Combretum apiculatum* (A8), *Combretum edwardsii* (A9), *Combretum petrophyllum* (A10), *Combretum albopunctatum* (A11), *Combretum microphyllum* (A12), *Combretum paniculatum* (A13), (B) *Combretum celestroides* subsp. *celestroides* (B1), *Combretum celestroides* subsp. *orientale* (B2), *Combretum padoides* (B3), *Combretum imberbe* (B4), *Combretum zeyheri* (B5), *Combretum mkuzense* (B6), *Combretum acutifolium* (B7), *Combretum oxystrachyum* (B8), *Combretum elaeagnoides* (B9), *Combretum hereroense* (B10), *Combretum collinum* subsp. *suluense* (B11), *Combretum bracteosum* (B12), *Combretum adenogonium* (B13), (C) *Quisqualis litorea* (C1), *Pteleopsis myrtifolia* (C2), *Terminalia brachystemma* subsp. *brachystemma* (C3), *Terminalia sericea* (C4), *Terminalia gazensis* (C5), *Terminalia sambesiaca* (C6), *Terminalia mollis* (C7), *Terminalia phanerophlebia* (C8), *Terminalia prunioides* (C9), *Terminalia brachystemma* subsp. *brachystemma* (C10), *Terminalia sericea* (C11), *Terminalia gazensis* (C12), *Terminalia brachystemma* subsp. *brachystemma* (C13), ursolic acid standard (S1), epicatechin standard (S2)

Combretaceae species. We are also able to predict, through HPTLC, which species are most likely to be active against specific pathogens. For example, the unknown compound (*m/z* 471) corresponding to Rf 0.71 displayed interesting activities. It was present in several of the extracts at different concentrations. The highest intensity bands were present in the extracts of *Combretum caffrum* (Figure 4, track A1), *Combretum krausii* (Figure 4, track A2), *Combretum nelsonii* (Figure 4, track A3), *Combretum vendae* (Figure 4, track A4) and *Combretum molle* (Figure 4, track A7). It is worthy to note that *Combretum krausii* displayed inhibition at Rf 0.71 against *Salmonella typhimurium*, whereas *Combretum woodii* did not. Inspection of the bands of the two species indicates that the intensity of the compound *m/z* 471 was low in *Combretum woodii*. That could be responsible for the inactivity of the extract towards *Salmonella typhimurium*. In *Combretum imberbe* (Figure 4, track B4), two compounds corresponding to *m/z* 471 were identified at Rf 0.69 and 0.71. These compounds were differentiated based on the colour of the bands. Imberbic acid was identified as a light purple band appearing at the slightly lower

Rf (0.69) than the other compound (Rf 0.71) with the dark purple colour (Figure 4, track B4). The activity of *Combretum imberbe* against *Salmonella typhimurium* did not result in broad inhibition zones as observed against *S. aureus* and *E. coli*. Fewer compounds contributed to the activity of *Combretum imberbe* against *Salmonella typhimurium*. It is possible that the unknown compound (Rf 0.71) with *m/z* 471 was responsible for the activity of *Combretum imberbe*, and not imberbic acid. A similar deduction was made for the activity against *B. cereus*, where broad inhibition zones were absent for *Combretum imberbe*. Although the compound (*m/z* 471; Rf 0.71) present in *Combretum kraussii* inhibited the activity of *Salmonella typhimurium*, the same was not observed against *B. cereus*. Similarly, the compound *m/z* 471 detected in *Combretum padiodes*, *Combretum bracteosum*, *Combretum microphyllum* and *Combretum molle* was active against *B. cereus*, but inactive towards *Salmonella typhi*. Consistency in inhibition was found only in the case of *Combretum zeyheri* and *Combretum imberbe*, in which compound *m/z* 471 (Rf 0.71) was active against both pathogens. A plausible explanation is that more than one compound with the molecular ion *m/z* 471 was present. Compound *m/z* 471 (Rf 0.71) was present in all the *Terminalia*, *Quisqualis* and *Pteleopsis* species. However, the *Terminalia* species selected for this study were not active in the bioautography analysis, suggesting that the inactivity is due to insufficient concentrations of active compounds, rather than their absence. The compound (*m/z* 471; Rf 0.71) was also detected in several *Combretum* species, although at low intensity. This observation suggests that compound *m/z* 471 is ubiquitous in South African Combretaceae species and that its antibacterial activity is concentration-dependent in the extract. Chemotaxonomically, the compounds corresponding to the molecular ions *m/z* 455, 471 and 487 are common to the Combretaceae species and contribute to the antibacterial activity. Although the UPLC–MS biochemometric model predicted that triterpenoids contributed to the activities of the species,[29] HPTLC provides further details relating to their chemotaxonomical relevance. In our previous study, *Combretum imberbe* was identified as the most active species. A similar observation was made in this study. From the HPTLC, the chemistry of *Combretum imberbe* was different to that of other species in the section *Hypocrateropsis*. It also differed from species in other sections, for example imberbic acid was only detected in *Combretum imberbe*. There is no available report of imberbic acid isolated or identified in other Combretaceae species. The number of triterpenoid bands visible in *Combretum imberbe* was higher than in any other species. The application of bioautography in this study clearly indicated that an additive effect of imberbic acid and its derivatives could be responsible for the low MIC values reported for *Combretum imberbe* (Table 1). The bioautography analysis also indicated that these multiple triterpenoids exhibited additive potential as indicated by the broad inhibition zones when tested against *S. aureus* and *E. coli*.

The compound corresponding to Rf 0.62 (jessic acid) was only detected in *Combretum elaeagnoides*. There are no available reports of jessic acid found in the leaves of any other Combretaceae species however, jessic acid 3-*O*-$\beta$-D-xylopyaranoside has been isolated from *Combretum molle* fruit.[37] This compound was active against all four pathogens, suggesting broad-spectrum potential. In our previous study,[29] the UPLC–MS model indicated that triterpenoids displayed broad-spectrum activities. In this study, jessic acid and imberbic acid (*m/z* 471) inhibited all four pathogens and could be described as possessing broad-spectrum activity. The concentration of the compound corresponding to Rf 0.69 was high in *Combretum apiculatum*, *Combretum elaeagnoides*, *Combretum hereroense* and *T. gazensis*, and displayed inhibition against *S. aureus* in the three *Combretum* species. The compound, identified as epicatechin, displayed selective activity towards *S. aureus*. The low intensity of epicatechin in *T. sericea* and *T. sambesciaca* explains the poor activity of their extracts against *S. aureus*.

The HPTLC profile and bioautography analysis suggest that flavonoids are the main antibacterial constituents of *Combretum acutifolium*, while jessic acid is the major antibacterial constituent of *Combretum elaeagnoides*. Without a laborious isolation process, bioautography coupled with HPTLC–MS provided insight into bioactive chemical constituents. The rapid screening and identification of compounds resulted in the identification of antibacterial markers (arjunic acid, ursolic acid and an unknown triterpenoid with *m/z* 471) that confer antibacterial activity to species belonging to South African Combretaceae. Furthermore, this study predicted the species or sections that are reliable sources of antibacterial compounds. For example, species belonging to the section *Angustimarginata* are rich sources of arjunic acid (*m/z* 487) and triterpenoid *m/z* 471, while *Combretum zeyheri* and *Combretum mkuzense* belonging to the sections Macrostigmatea and Spathuipetala, respectively, are rich source of ursolic acid. The compound has been previously isolated from *Combretum zeyheri* leaves but was not active against strains of *Candida albicans*.[38] The study also suggests the presence of isomers, since a compound identified in different species displayed varying inhibitory activities. This must be validated through UPLC–MS analysis. This study has demonstrated that bioautography coupled with HPTLC–MS is a useful tool for the direct identification of compounds with antibacterial activities within South African Combretaceae species.

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## DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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## SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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