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Original Research Article

Serratiochelins A and B from *Serratia marcescens* show xenosiderophoric characteristics towards *Acinetobacter baumannii* and *Mycobacterium tuberculosis*

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Abstract

Purpose: *To evaluate the xenosiderophoric properties of siderophores produced by Serratia marcescens towards Acinetobacter baumannii, Staphylococcus aureus and Mycobacterium tuberculosis.*

Methods: *A non-pigmented strain of S. marcescens was isolated from soil after cultivation in iron-limited LB medium. The isolate was identified using both biochemical and 16S rDNA molecular phylogenetic analyses. The bacterial secondary metabolites were extracted after solid state fermentation in sterile rice medium. The extract was separated using chromatography, and the resulting compounds were analyzed by mass spectrometry and nuclear magnetic resonance spectroscopy. The iron-chelating, growth-promoting and cytotoxic activities of the compounds were determined using standard protocols.*

Results: *Two siderophore compounds (serratiochelins A and B) were isolated from the fermentation extract of S. marcescens. Characteristic of siderophores, serratiochelins A and B exhibited varying degrees of iron-chelating activities. The compounds displayed xenosiderophoric properties by supporting the growth of A. baumannii and M. tuberculosis in iron-limited media. In addition, the siderophores displayed cytotoxic activity against human cells, with serratiochelin A showing the higher activity with IC50 of 3.20 and 6.26 µM against THP-1 and HEK-293 cells, respectively.*

Conclusion: *This study demonstrates the isolation of serratiochelins A and B from a soil-derived non-pigmented strain of S. marcescens. The siderophores support the growth of A. baumannii and M. tuberculosis, and thus, have prospects for development as sideromycins against these multidrug resistant (MDR) organisms.*

Keywords: *Serratia marcescens, Siderophores, Xenosiderophores, Serratiochelin, Sideromycins, Acinetobacter baumannii, Mycobacterium tuberculosis*

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INTRODUCTION

Siderophores are small natural molecules with a high affinity for iron that are secreted by bacteria and are critical for bacterial survival, particularly in iron-deficient environments. Bacterial iron acquisition systems include the biosynthesis of siderophores and their associated transport systems located in the inner and outer membranes. This ability of bacteria to actively transport an essential nutrient like iron, either generally or specifically, indicates that the mechanism may be used to create vectors for the general or specific internalization of other agents, such as antibiotics, into bacteria [1,2].

While some bacteria express outer membrane-bound receptors and transport proteins that are specific for their own or some very unique sets of siderophores, others will utilize siderophores produced by a different bacterial species. Bacterial utilization of xenosiderophores (siderophores produced by other organisms) may have evolved to reduce the amount of energy spent on siderophore biosynthesis while promoting bacterial survival in iron-deficient environments. The ability of bacteria to actively transport an essential nutrient such as iron suggests that the process could be used to develop vectors for the internalization of other agents, especially antibiotics, into bacteria [2].

Siderophores are promising candidates for the synthesis of siderophore-antibiotic conjugates (also referred to as sideromycins). Sideromycin consists of an antibiotic covalently linked by a ‘tether' to a siderophore. These sideromycins are actively transported into bacteria by their iron transport machinery, which accepts siderophores as substrates. The sideromycin strategy has recently generated interest due to the possibility of overcoming permeability issues and facilitating antibiotic transport across the bacterial cell wall, thereby aiding bacterial death. This concept has been validated clinically and addresses a key issue of Gram-negative pathogens, the impaired translocation into the cell [3]. Several naturally occurring sideromycins, such as albomycins and salmycins, have been shown to enter the targeted bacteria via the iron transport system and are thus very effective antibacterial agents [4]. A number of synthetic siderophore-drug conjugates have also been reported, including "cefiderocol," a novel siderophore-cephalosporin sideromycin that targets Gram-negative bacteria, including strains with carbapenem resistance [5].

This study evaluates the xenosiderophoric-property of sideropohores isolated from a strain of soil-derived *S. marcescens* with regard to supporting the growth of *A. baumannii*, *Staphylococcus aureus* and *M. tuberculosis* in an iron-deficient environment, and their prospects for the development of sideromycins against these human pathogens.

EXPERIMENTAL

Bacterial isolation and identification

A soil sample collected from Wildpark Grafenberger Wald, Rennbahnstraße, Düsseldorf, Germany, was inoculated into sterile iron-limited LB medium and incubated for 30 ºC for 14 days. This was to favor the survival and growth of siderophore-producing bacterial strains with the ability to sequester iron from the environment. About 500 µL of broth culture was then transferred to sterile tubes which were centrifuged at 500 rpm for 5 min to remove debris. The supernatant was then transferred to sterile M9 solid medium for bacterial isolation. The M9 plates were incubated at 30 ºC for 72 h. The isolate was identified as a non-pigmented strain of *S. marcescens* using both biochemical and 16S rDNA molecular phylogenetic analyses. The genomic sequence of the bacterial isolate was deposited in GenBank under the accession no. MT510350.

Fermentation, extraction and isolation of siderophores

The bacterial isolate was subjected to solid state fermentation in 2 sterile Erlenmeyer flasks containing rice medium supplemented with 1 % tryptone, 0.5 % yeast extract and 0.5% NaCl. The fermentation flasks were incubated at 37ºC for 21 days. Using ethyl acetate (EtOAc), the secondary metabolites of the bacterium were extracted and concentrated under reduced pressure. Initial purification of the total crude ethyl acetate extracts from both fermentation flasks (1.26 g) was performed using fractionation with vacuum liquid chromatography (VLC) on silica gel using a gradient elution (100:0, 95:5, 95:5, 95:5, 90:10, 80:20 and 0:100) of 300 mL dichloromethane (DCM) – methanol (MeOH) to yield 7 fractions (F1 – F7) respectively. Compound **1**, the most abundant compound in the extract, was isolated from fractions F3 (246.4 mg), F4 (143.8 mg) and F7 (54.6 mg) and assigned the codes (SM-3-2; 9.2 mg), (SM-4-1; 1.2 mg) and (SM-7-1; 0.8 mg) respectively. Compound **2** (code: SM-5-2, weight: 1.6 mg) was isolated from fraction F5 (208.7 mg). Isolation, purification and characterization of the compounds from the fractions were achieved using semi-preparative HPLC, mass spectral

(MS) analysis, and nuclear magnetic resonance (NMR) spectroscopy.

General analytical procedures

Nuclear magnetic resonance (NMR) spectroscopic analyses of the isolated compounds were performed in deuterated methanol and or dimethyl sulfoxide using a Bruker Avance DMX 600 spectrometer (Bruker BioSpin, Germany). The NMR spectra were referenced relative to the residual solvent signals. For mass spectral analysis, ESIMS and HRESIMS were measured with a UHR-QTOF maXis 4G (Bruker Daltonik, Germany) mass spectrometer. A Dionex P580 system connected to a P580A LPG pump, a photodiode array detector (UVD340s, Dionex Softron, Germany), and a separation column (125 x 4 mm) pre-filled with Eurosphere-10 C18 (Knauer, Germany) was used for analytical HPLC analysis, with MeOH:H_2O mixtures as the gradient solvent system. For semi-preparative HPLC, a Merck-Hitachi HPLC system with a UV detector (L-7400), pump (L-7100), and a Eurosphere column (100 C18, 300 x 8 mm, Knauer, Germany) was used at a flow rate of 5.0 mL/min with gradient MeOH:H_2O mixtures as the mobile phase. Vacuum Liquid chromatography (VLC) was carried out using silica gel 60 (230–400 mesh, Merck, Germany). Pre-coated TLC Silica gel 60 F_{254} plates (20×20 cm, Merck, Germany) were used to monitor fractions under UV detection (Camag UV cabinet, Germany) at 254 and 366 nm. A P-2000 polarimeter (Jasco, Germany) was used to measure the optical rotation of the isolated compounds in MeOH. Distilled solvents were used for column chromatography, while spectral-grade solvents were used for spectroscopic measurements.

Measurement of Fe^{2+} chelating ability

The Fe^{2+} chelating ability of the isolated compounds was evaluated using a previously described method [6]. A 2-fold serial dilution of the tested compounds (31.25, 62.5, 125, 250, 500, and 1000 µg/mL) was prepared by dissolving in distilled water. A volume of 2 µL of 2 mM $FeCl_2$ and 4 µL of 5 mM ferrozine were added to 100 µL of each sample. After a reaction time of 10 min, the absorbance of the resulting solutions was measured at 562 nm using a Tecan Infinite 200pro microplate reader. A complex of Fe^{2+}/ferrozine shows a strong absorbance at 562 nm, while a test sample with a high ferrous ion-chelating ability exhibit a low absorbance. Nanopure water (100 µL), to which was added 2 µL of 2 mM $FeCl_2$ and 4 µL of 5 mM ferrozine, was used as the negative control. Nanopure water (100 µL) was used as blank. The ferrous ion-chelating ability was calculated as follows:

$$\text{Fe2+ chelating ability (\%)} = \frac{[A_C - (A_S - A_\theta]}{A_C} \times 100$$

where A_C was the absorbance of the control, A_S was the absorbance of the sample, and A_B was the absorbance of the blank.

Growth promotion assay

The xenosiderophoric growth promoting activities of the isolated siderophores were determined at concentrations of 100 to 0.049 µM on cultures of *Staphylococcus aureus* (ATCC 29213), *Acinetobacter baumannii* (BAA 1605) and *Mycobacterium tuberculosis* (H37Rv). The Clinical and Laboratory Standards Institute (CLSI) micro-dilution method in Müller Hinton broth [7] was employed in the assay of *S. aureus* and *A. baumannii*. Ciprofloxacin (100 to 0.049 µg/mL) and DMSO (100% v/v) were used as positive and negative controls respectively. The method described by Daletos *et al* [8] was employed in the assay of *M. tuberculosis*. Mycobacterial cells were grown aerobically in a humidified atmosphere at 37 °C and 5% CO_2 in Middlebrook 7H9 media supplemented with glycerol (0.5% v/v), tyloxapol (0.05% v/v), and ADS enrichment (10% v/v). The mycobacterial cells were standardized (OD 600 nm ~0.01) and seeded in a 96-well round-bottom microtiter plates at 1 × 10^5 cells per well and incubated with test substances in a total volume of 100 µL for 5 days. Rifampicin (0.049 to 100 µg/mL) and DMSO (100% v/v) were used as positive and negative controls respectively. To assess cell viability, 10 µL of 100 µg/mL resazurin solution (Sigma-Aldrich) was added into each well and the plates were incubated at 37 °C for 8 h. The cells were fixed by addition of formalin (5 % v/v) and incubating at room temperature for 30 min. Fluorescence was then measured using a Tecan Infinite 200pro microplate reader (excitation 540 nm, emission 590 nm). Residual growth was calculated relative to the sterile medium (0% growth) and DMSO-treated (100% growth) controls.

Cytotoxicity assay

Cytotoxicity studies were conducted on compounds **1** and **2** against human monocytic leukemia cell line (THP-1) and human embryonic kidney epithelial cell line (HEK-293) using a previously described method [9]. THP-1 cells were cultured in RPMI 1640 medium containing 10 % fetal bovine serum (FBS). The HEK-293 cells were cultivated in EMEM medium supplemented with 2 mM L-glutamine, 1 % non-

essential amino acids, 1mM sodium pyruvate, and 10% (v/v) FBS. The cells were incubated at 37 ºC in a humidified atmosphere of 5 % CO_2 for 5 days. The cells were then suspended and adjusted to a density of 1×10^6 cells/mL. Thereafter, cells were then seeded into a 96-well cell culture plate (suspension cells plate and adherent cells plate were used for THP-1 and HEK-293 cells respectively), in a total volume of 100 µL containing 2-fold serial dilutions of the tested compounds in a concentration range of 0.78 to 100 µM. A volume of 2 µL of Triton X-100 (100 % v/v) was used as positive control. The plates were incubated at 37 °C in a humidified atmosphere of 5 % CO_2 for 48 h. A volume of 10 µL resazurin solution (100 µg/mL) was then added to each well, and the plates were further incubated for 4 h. Fluorescence was measured using a Tecan Infinite 200pro microplate reader (excitation 540 nm, emission 590 nm). Residual growth was calculated relative to non-inoculated (0% growth) and untreated (100% growth) controls, respectively.

Statistical analysis

All measurements in the bioassays (Fe^{2+} chelating ability, growth promotion and cytotoxicity assays) were performed in triplicate, and the mean values were calculated and recorded.

RESULTS

Chromatographic separation and spectroscopic analyses of the bacterial extract resulted in the isolation two known siderophore compounds (serratiochelins A and B) with iron-chelating and growth-promoting activities. The chemical structures of the isolated compounds are shown in Figure 1. Results of the ferric ion-chelating capability, xenosiderophoric growth-promotion, and cytotoxicity assays of the siderophores are presented in Figure 2, Figure 3 and Figure 4, respectively.

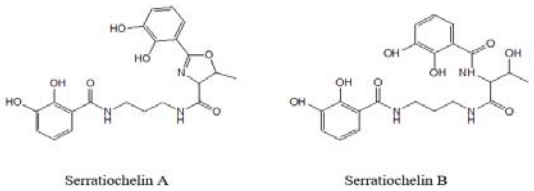

Figure 1: Structures of the isolated siderophores

Serratiochelin A

Compound **1** (SM-4-1) showed UV (MeOH) λ_{max} at 253.7 and 315.3 nm. The ESIMS displayed pseudo-molecular ions at m/z 430 $(M+H)^+$ revealing a molecular weight of 429 g/mol. The ^1H NMR (600 MHz, Methanol-d_4) spectrum showed the following signals: δ 7.20 (ddd, *J* = 8.0, 3.3, 1.4 Hz, 4H), 6.98 (dd, *J* = 7.9, 1.5 Hz, 1H), 6.93 (dd, *J* = 7.8, 1.5 Hz, 1H), 6.77 (t, *J* = 7.9 Hz, 1H), 6.72 (t, *J* = 8.0 Hz, 1H), 4.90 (dd, *J* = 7.5, 6.3 Hz, 1H), 4.48 (d, *J* = 7.5 Hz, 1H), 3.45 (t, *J* = 6.7 Hz, 2H), 3.38 – 3.32 (m, 3H), 1.83 (p, *J* = 6.7 Hz, 1H), 1.57 (d, *J* = 6.3 Hz, 2H). The optical rotation $(\alpha)_D^{20}$ = -128.75º (c 0.15, MeOH) was recorded. Interpretation of the ^1H-NMR and mass spectral data suggested the molecular formula $C_{21}H_{23}N_3O_7$. Thus, the compound was identified as the previously reported siderophore serratiochelin A, also known as serratiochelin, or serranticin, as confirmed by previous reports [6,10].

Serratiochelin B

Compound **2** (SM-5-2) showed UV (MeOH) λ_{max} at 249 and 312 nm. The ESIMS displayed pseudo-molecular ions at m/z 448 $(M+H)^+$ and 470 $(M+Na)^+$ revealing a molecular weight of 447 g/mol. The ^1H NMR (600 MHz, methanol-d_4) spectrum showed the following signals: δ 7.38 (dd, *J* = 8.1, 1.5 Hz, 1H), 7.21 (dd, *J* = 8.1, 1.4 Hz, 1H), 6.96 (dd, *J* = 7.8, 1.5 Hz, 1H), 6.92 (dd, *J* = 7.8, 1.4 Hz, 1H), 6.75 (t, *J* = 7.9 Hz, 1H), 6.71 (t, *J* = 8.0 Hz, 1H), 4.51 (d, *J* = 3.5 Hz, 1H), 4.35 (qd, *J* = 6.4, 3.5 Hz, 1H), 3.58 – 3.50 (m, 2H), 3.44 (td, *J* = 6.7, 2.4 Hz, 2H), 1.81 (p, *J* = 6.7 Hz, 2H), 1.27 (d, *J* = 6.4 Hz, 3H). The optical rotation $(\alpha)_D^{20}$ = 22.16º (c 0.2, MeOH) was recorded. Interpretation of the ^1H-NMR and mass spectral data suggested the molecular formula $C_{21}H_{25}N_3O_8$. Thus, the compound was identified as the siderophore serratiochelin B, previously reported by Seyedsayamdost *et al* [10].

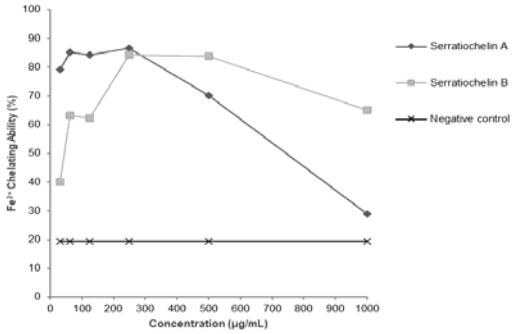

Figure 2: Fe^{2+} chelating abilities of the siderophores. The compounds exhibited varying degrees of iron-chelating activities. Highest iron-chelating activity was recorded for the siderophores at 250 µg/mL, with serratiochelins A and B showing ferrous-chelating ability of 86.64 and 84.33 %, respectively

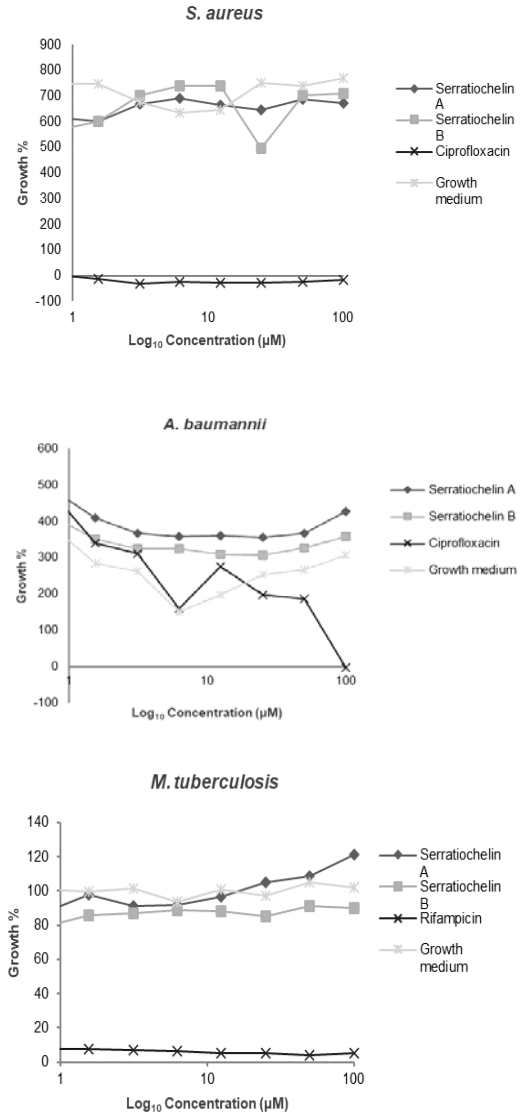

Figure 3: Assessment of xenosiderophoric growth-promoting activities of the siderophores on *S. aureus, A. baumannii* and *M. tuberculosis.*

Except for *S. aureus* which grew better in medium without the siderophores, the two siderophores displayed varying abilities to support the growth of *A. baumannii* and *M. tuberculosis* in iron-limited media. Relative to the growth medium with percent growth ranging from 150.1 – 383.5%, serratiochelin A significantly enhanced the growth of *A. baumannii* with highest activity (485.5%) recorded at 0.78 µM. Serratiochelin B showed growth-promoting activity of 410.8 % at 0.78 µ. Also, serratiochelin A at 25 – 100 µM enhanced the growth of *M. tuberculosis* with highest activity of 121.0% recorded at 100 µM, relative to the growth medium with percent growth that ranged from 93.3 – 104.9 %. Growth-promotion of *M. tuberculosis* was not observed for serratiochelin B.

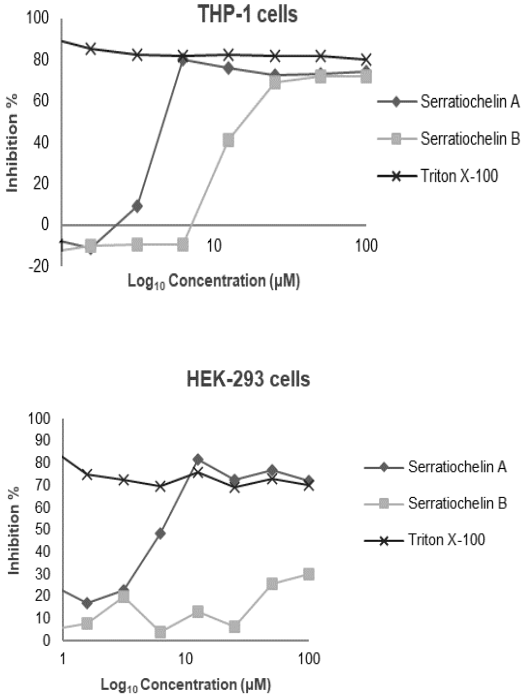

Figure 4: Cytotoxicity of the siderophores against THP-1 and HEK-293 cell lines. Serratiochelin A displayed cytotoxic activity against the human cells with IC_{50} of 3.20 µM (THP-1 cells) and 6.26 µM (HEK-293 cells). Serratiochelin B showed lower cytotoxicity against THP-1 and HEK-293 cells, with IC_{50} of 20.00 and 45.23 µM, respectively

DISCUSSION

Serratia marcescens is a Gram-negative bacterium of the Enterobacteriaceae family. *S. marcescens* is the most studied member of the genus *Serratia*, and it is known to produce a red prodigiosin pigment. The bacterium can successfully adapt to different ecological niches and colonize various surfaces, including human tissues, where it can cause infections of the urinary tract, respiratory tract, and surgical wounds, posing an increasing public health problem. [11,12]. *S. marcescens* is intensely studied with regard to its biosynthetic pathways and biological activities of its metabolites. Members of this genus are known to produce a variety of secondary metabolites including prodigiosin, serratamolide and a carbapenem [13-16]. Extracts from the fermentation broth of a species of *Serratia* showed antibiotic activity against species of *Mycobacterium* [17].

Among their many secondary metabolites, the human opportunistic pathogen *S. marcescens* secretes siderophores to enable its growth under iron-limiting conditions. The siderophores are involved in iron chelation (as well as the transport of some other metals), oxidative stress protection, and molecular signaling [11]. Three different types of siderophores have been described in enterobacteria: catecholates (enterobactin, salmochelins, and serratiochelins), hydroxamates (ferrioxamines) and mixed-ligand-type siderophores (aerobactin, and yersiniabactin) [1,18]. The serratiochelins are the most characterized siderophores produced by *S. marcescens*. Serratiochelins A, B, and C have been isolated and identified from this bacterium [6,10].

This study describes the isolation of two previously reported siderophores, serratiochelins A and B (Figure 1) from *S. marcescens*. According to Drechsel and Jung [19], for a secondary metabolite to be classified as siderophore, three conditions must be met: (1) iron-regulated biosynthesis, that is, the siderophores are only produced in an iron-deficient condition; (2) ferric ion-chelating capability; and (3) active transport through the cell membrane. In this study, the biosynthesis of the two siderophore compounds in an iron-deficient medium confirms the first condition. Results of the ferric ion-chelating capability test showed that the compounds exhibited varying degrees of iron-chelation, thereby confirming the second condition. Finally, the ability to support bacterial growth in an iron-deficient medium confirms the third condition of active transport through the cell membrane.

With the exception of *S. aureus,* which was inhibited, the siderophores isolated from *S. marcescens* in this study exhibited varying degrees of xenosiderophoric growth-promoting activity on *A. baumannii* and *M. tuberculosis*. The siderophores readily promoted the growth of *A. baumannii,* with serratiochelin A showing highest growth-promoting activity. In addition, relative to the growth medium and compared to serratiochelins B, serratiochelin A enhanced the growth of *M. tuberculosis.* A similar study by Matzanke *et al* [20] showed that serratiochelin A supported the growth of *Mycobacterium* spp. in an iron-deficient medium.

As observed in this study, the two siderophores serratiochelins A and B were utilized by the test bacteria (*A. baumannii* and *M. tuberculosis*) to support their growth and metabolism in an iron-deficient medium. It is believed that this ability to utilize xenosiderophores aids bacterial survival in competitive iron-deficient environments by reducing the amount of energy spent on siderophore biosynthesis [2]. The siderophores described in this study are indeed possible candidates for developing sideromycins and can be used as a probe to investigate sideromycin uptake in MDR organisms such as the carbapenem-resistant *A. baumannii* and the famously pathogenic *M. tuberculosis*.

Apart from being essential for bacterial growth and adaptation in iron-deficient environments, siderophores have been linked to a variety of biological activities and applications. These include antioxidant and antitumor activities [6]. The siderophores isolated in this study displayed cytotoxic properties, with serratiochelin A showing stronger activity with IC_{50} of 3.20 µM and 6.26 µM against human THP-1 and HEK-293 cells respectively.

Siderophores have also been reported to have applications in the treatment and management of diseases related to iron overload [6,21]. Desferrioxamine B (Desferal™), a major siderophore of *Streptomyces pilosus*, is used to treat diseases caused by iron intoxication or overload, as well as aluminium toxicity (which can occur in dialysis patients) [22,23]. Parabactin, a siderophore isolated from *Paracoccus dentrificans,* was reported to be nearly 300 % more effective than desferrio-xamine, which is a clinically accepted agent for iron decorporation [23]. Desferrioxamine B has also been shown to be active against *P. falciparum in vitro* as well as *in vivo* [23].

CONCLUSION

The results of this study demonstrate the isolation of two siderophores (serratiochelins A and B) from a soil-derived non-pigmented strain of *S. marcescens*. The siderophores promote the growth of *A. baumannii* and *M. tuberculosis* in iron-limited media, and thus have the potential to be developed as sideromycins against these MDR organisms.

DECLARATIONS

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Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. RK conceptualized and supervised the study. PME, VS, TS, and LW managed the laboratory analyses. LvG and AK participated in the bioassays. PME, VS, LvG, MF, CCA, COE and FBCO participated in data analyses and validation, literature searches, and preparation of the first draft of the manuscript. All authors read and approved the final manuscript.

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