



## *Cladosporium sphaerospermum* extract inhibits quorum sensing associated virulence factors of *Serratia marcescens*

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### ABSTRACT

*Serratia marcescens* is now becoming a propensity for its highly antimicrobial-resistant clinical infections. Currently, it provides a novel strategy to prevent and control microbial infection by regulating *S. marcescens* quorum sensing (QS). Deep-sea-derived fungi are rich in QS bioactive constituents. In this work, the extracts from *Cladosporium sphaerospermum* SCSGAF0054 showed potent QS-related virulence factors and biofilm-inhibiting activities against *S. marcescens* NJ01. The swimming motility and multiple virulence factors such as prodigiosin, exopolysaccharide (EPS), lipase, protease and hemolysin were moderately inhibited by the extracts at varied concentrations. The confocal laser scanning microscope (CLSM) and scanning electron microscope (SEM) images revealed that *C. sphaerospermum* extracts moderately arrested biofilm formation and cell viability. Further, real-time quantitative PCR (RT-qPCR) analysis revealed that expressions of genes associated with virulence factors, *flhD*, *fimA*, *fimC*, *bsmA*, *bsmB*, *pigA*, *pigC*, and *shlA*, were significantly down-regulated compared with control. In addition, the extracts combined with imipenem inhibited the QS system of *S. marcescens* NJ01, disrupted its preformed biofilm, released the intra-biofilm bacteria and killed the bacteria gradually. Therefore, the extracts combined with imipenem can partially restore bacterial drug sensitivity. These results suggest that the extracts from SCSGAF0054 effectively interfere with the QS system to treat *S. marcescens* infection alone or combining with classical antimicrobial drugs.

### 1. Introduction

*Serratia marcescens* is one clinical pathogen that can cause lung and urinary tract infections and septicemia when the body's immune function decreases [1]. It is also a pathogen of many plants, causing plant diseases such as watermelon by clinging to plant tissue [2]. With the abuse or misuse of antimicrobial drugs, the resistance of *S. marcescens* to many common antimicrobial drugs is increasing. Carbapenem antimicrobial drugs are commonly used to treat infections of *S. marcescens* [3]. However, the resistance to carbapenem antimicrobial drugs was more serious, which caused the infection rate of the bacteria to increase gradually. Therefore, new alternative therapies and prevention strategies are urgently needed to update or replace antimicrobial treatments. Quorum sensing (QS) and biofilm inhibition may be one novel

alternative strategy to deal with such drug-resistant troubles.

Deep-sea-derived fungi are relatively untapped biological resources for discovering bioactive novel natural products. In the past decades, about 1 000 new natural products have been extracted from deep-sea-derived fungi, including terpenes, alkaloids, mangosteen-derived metabolites, lipids, polyketones and nonribosomal peptides [4]. Some of these compounds have antimicrobial and anticancer activities. Since the first quorum sensing inhibitor (QSI) was isolated from one red algae, some QSIs were developed in marine organisms [5]. QSIs have been found in the deep-sea-derived fungi *Sarocladium*, *Khuskia*, *Fusarium* and so on [6]. *Cladosporium* from the deep sea is of great interest for its ability to produce many metabolites, including macrolides, alkaloids, tetramic acid derivatives and so on [7]. These metabolites have various biological activities such as antimicrobial, antiviral, insecticidal, etc [8].

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Till now, cladodionen isolated from *Cladosporium sphaerospermum* Z148 has an anti-quorum sensing activity against *Pseudomonas aeruginosa* [9]. However, no reports of *C. sphaerospermum* against *S. marcescens* NJ01 have been found. Hence, the present investigation aims to evaluate the inhibitory effects of *C. sphaerospermum* SCSGAF0054 extracts against the QS-associated virulence factors and biofilm formation of *S. marcescens* NJ01.

## 2. Results

### 2.1. LC-MS profiling of *C. sphaerospermum* SCSGAF0054 extracts

The LC-MS analysis of *C. sphaerospermum* SCSGAF0054 extracts identified 1 057 compounds in the mzCloud database. Among these, 498 compounds exhibited high degree of similarity to the mzCloud database, with a score greater than 60.

Further, cyclo(L-Phe-L-Pro), 3-Phenyllactic acid, pyridoxine, anthranilic acid, thymine and benzoic acid were identified at retention time (Rt) of 12.1 min, Rt 11.5 min, Rt 1.5 min, Rt 10.0 min, Rt 4.7 min and Rt 10.1 min, respectively (Fig. 1).

The *C. sphaerospermum* SCSGAF0054 extract blocks the formation of prodigiosin, which is regulated by quorum sensing (QS) (Fig. 2).

### 2.2. Determination of minimum inhibitory concentrations (MICs) and growth profiles

The MIC of the extracts against *S. marcescens* NJ01 was 800 µg/mL, and the chosen concentration must be lower than the MIC to confirm that it did not inhibit bacterial growth. The result of the bacterial growth profile is represented in Fig. 3. The extracts had no effect on bacterial growth in the range of 50–400 µg/mL, so the concentrations were chosen for further studies. The FICI was 1.5, between 0.5 and 4, indicating that the extracts had no interaction with imipenem.

### 2.3. Inhibition of virulence factors

Fig. 4A,-E demonstrates that 400 µg/mL of the extracts inhibited the synthesis of prodigiosin (57%), protease (46%), lipase (37%), hemolysin

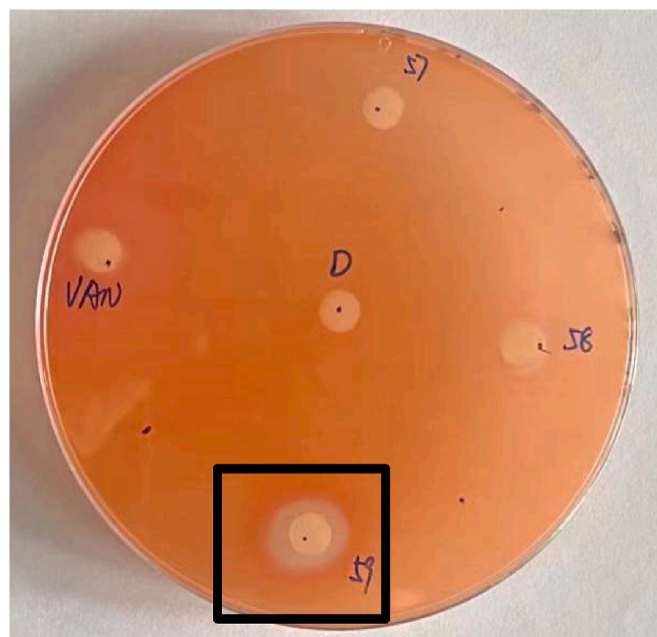


Fig. 2. Anti-quorum sensing activity of *C. sphaerospermum* extract against *S. marcescens* NJ01.

(37%) and exopolysaccharides (50%), respectively. Simultaneously, the inhibition rates of positive control vanillic acid (VNA) against prodigiosin, protease, lipase, hemolysin, and exopolysaccharides were 25%, 31%, 30%, 34%, and 14%, respectively, demonstrating that the extracts were more effective than the positive control.

### 2.4. Inhibition of swimming motility

The swimming ability of *S. marcescens* NJ01 was reduced after being treated with *C. sphaerospermum* extracts. The diameter of swimming motility was decreased (1.105 cm) when treated with 400 µg/mL of

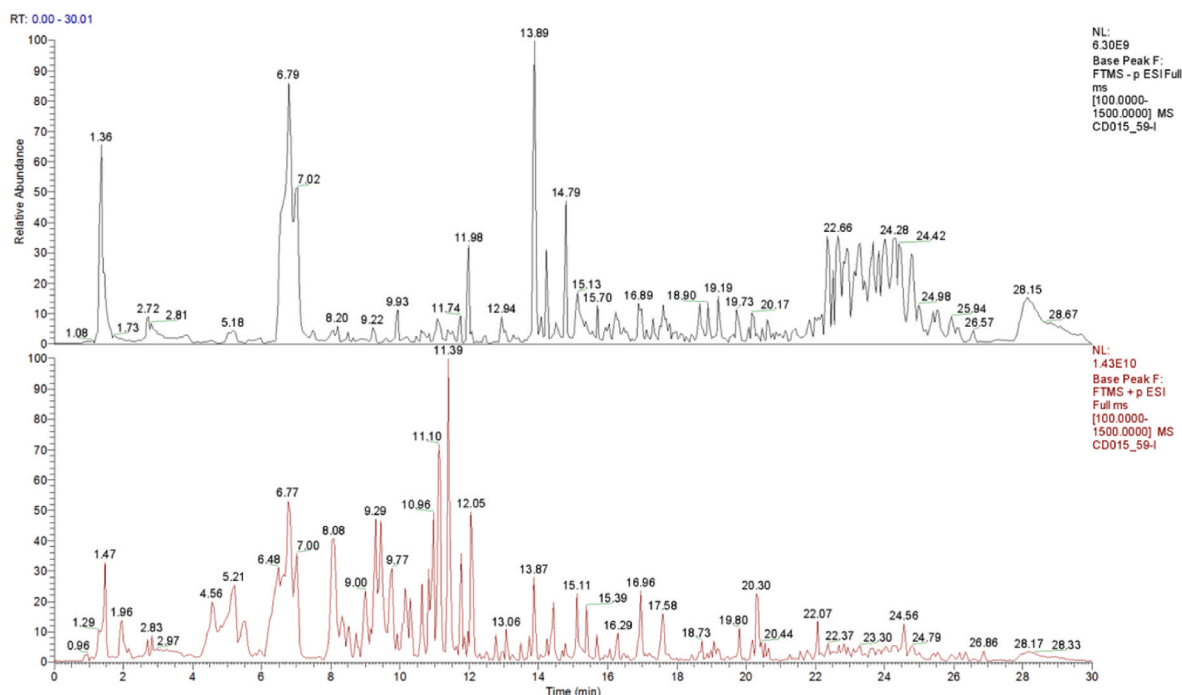


Fig. 1. Total ion chromatograms of the extracts by LC-MS.

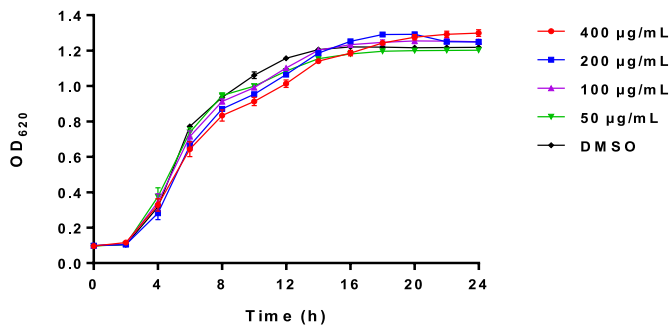


Fig. 3. Effects of the extracts against the *S. marcescens* NJ01 growth at sub-MIC levels. DMSO served as the negative control.

extracts. The swimming motility inhibition was observed at 53% (Fig. 5).

### 2.5. Inhibition of biofilm formation

The biofilm inhibition ratio for extracts at 400 µg/mL concentration was recorded as 50% (Fig. 6A). Similarly, the cell survival rate decreased by 44% when treated with 400 µg/mL of *C. sphaerospermum* extracts.

Additionally, CLSM and SEM observations have been performed to analyze the changes in the architecture of biofilms treated with the extracts. The CLSM images revealed that *C. sphaerospermum* extracts reduced and loosened biofilms, but the control image was uniformly dispersed (Fig. 7). Similarly, SEM images revealed that DMSO-exposed biofilms were dense and evenly distributed, whereas biofilms treated with variable concentrations of extracts loosened and disintegrated

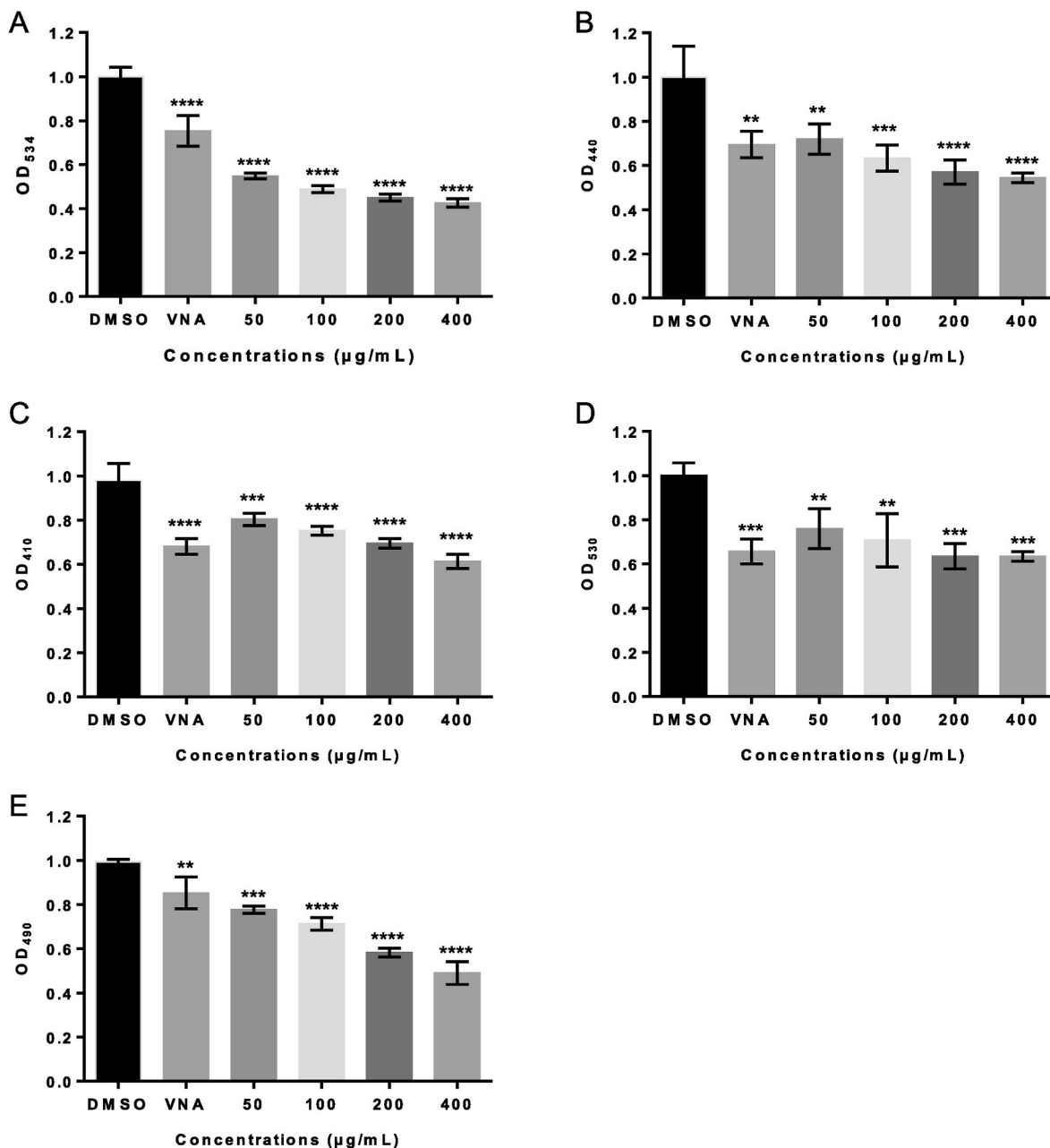
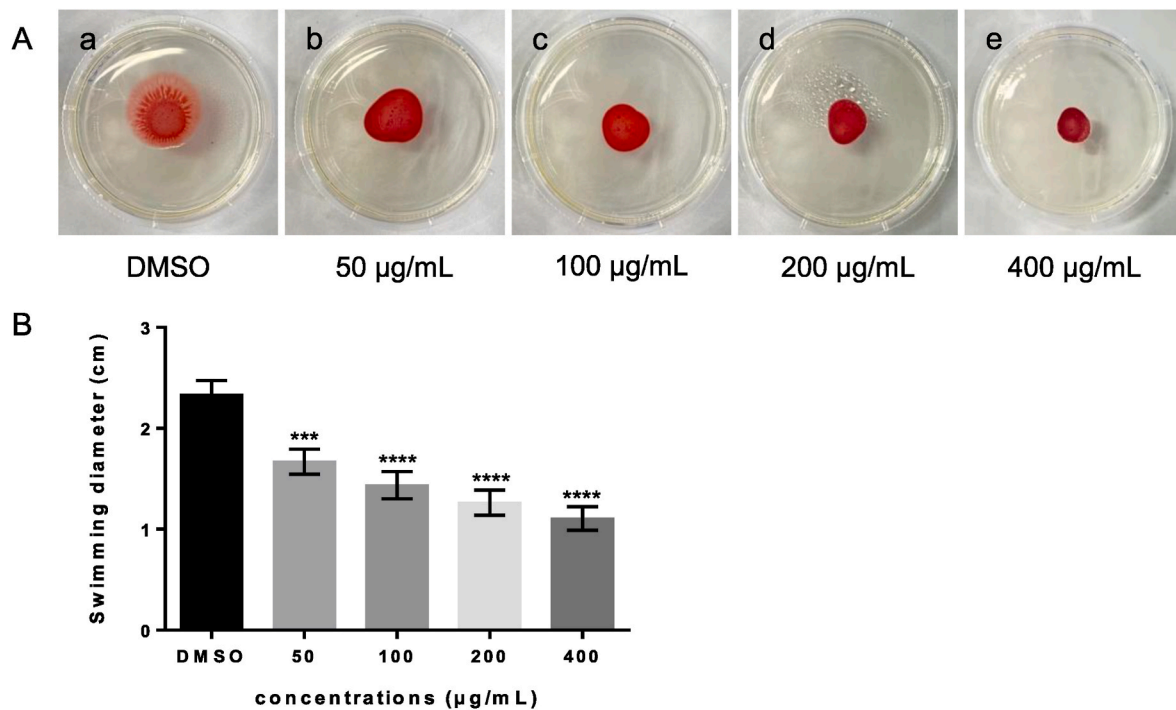
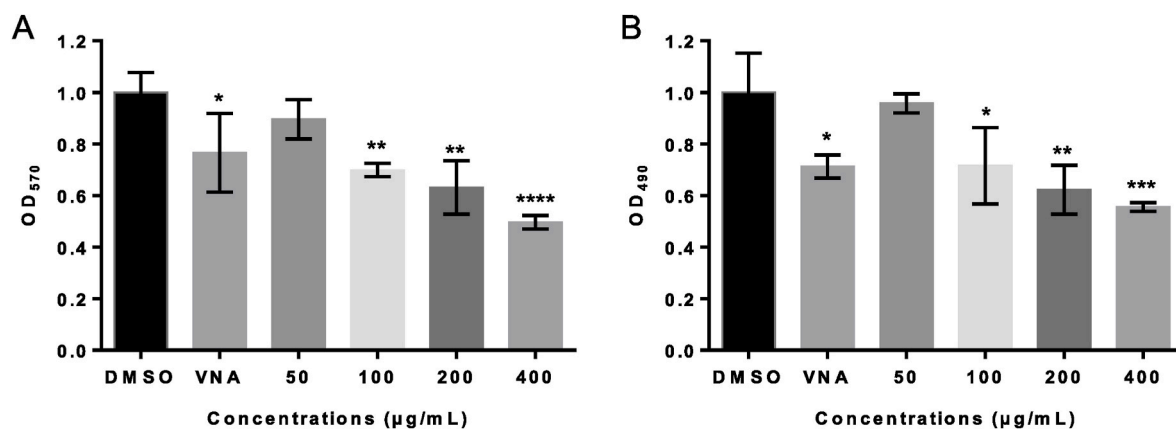


Fig. 4. Inhibition rates of the extracts on virulence factors of *S. marcescens* NJ01. (A) Prodigiosin, (B) Protease, (C) Lipase, (D) Hemolysin and (E) Exopolysaccharides. Data shown are mean ± standard deviation (n = 3). One-way ANOVA was used to assess statistical significance. \**p* < 0.05, \*\**p* < 0.01, \*\*\**p* < 0.001 and \*\*\*\**p* < 0.0001.



**Fig. 5.** Assessment of swimming motility inhibition of *S. marcescens* NJ01 treated with *C. sphaerospermum* extracts. (A) Swimming motility test (B) The measurement of swimming motility diameter (a) DMSO (b) 50 µg/mL extracts (c) 100 µg/mL extracts (d) 200 µg/mL extracts (e) 400 µg/mL extracts. Data shown are mean  $\pm$  standard deviation (n = 3). One-way ANOVA was used to assess statistical significance. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  and \*\*\*\* $p < 0.0001$ .



**Fig. 6.** Antibiofilm activity of *C. sphaerospermum* extracts against *S. marcescens* NJ01. (A) Crystal violet, (B) MTT. Data shown are mean  $\pm$  standard deviation (n = 3). One-way ANOVA was used to assess statistical significance. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  and \*\*\*\* $p < 0.0001$ .

(Fig. 8).

## 2.6. Damage of preformed biofilm in combination with imipenem

As illustrated in Fig. 9, treatment with imipenem alone had no significant eradication effect on the preformed biofilm, whereas the combination of 0.2 µg/mL of imipenem and 400 µg/mL of the extracts substantially eradicated the preformed biofilm by 45%. In addition, CLSM was used to observe changes in biofilms treated with the combination of extracts and 0.2 µg/mL of imipenem. CLSM analysis revealed that the biofilm treated with imipenem alone was dense and evenly distributed, whereas the biofilms were reduced, flattened, and loosened after exposure with 400 µg/mL of the extracts and 0.2 µg/mL of imipenem (Fig. 10).

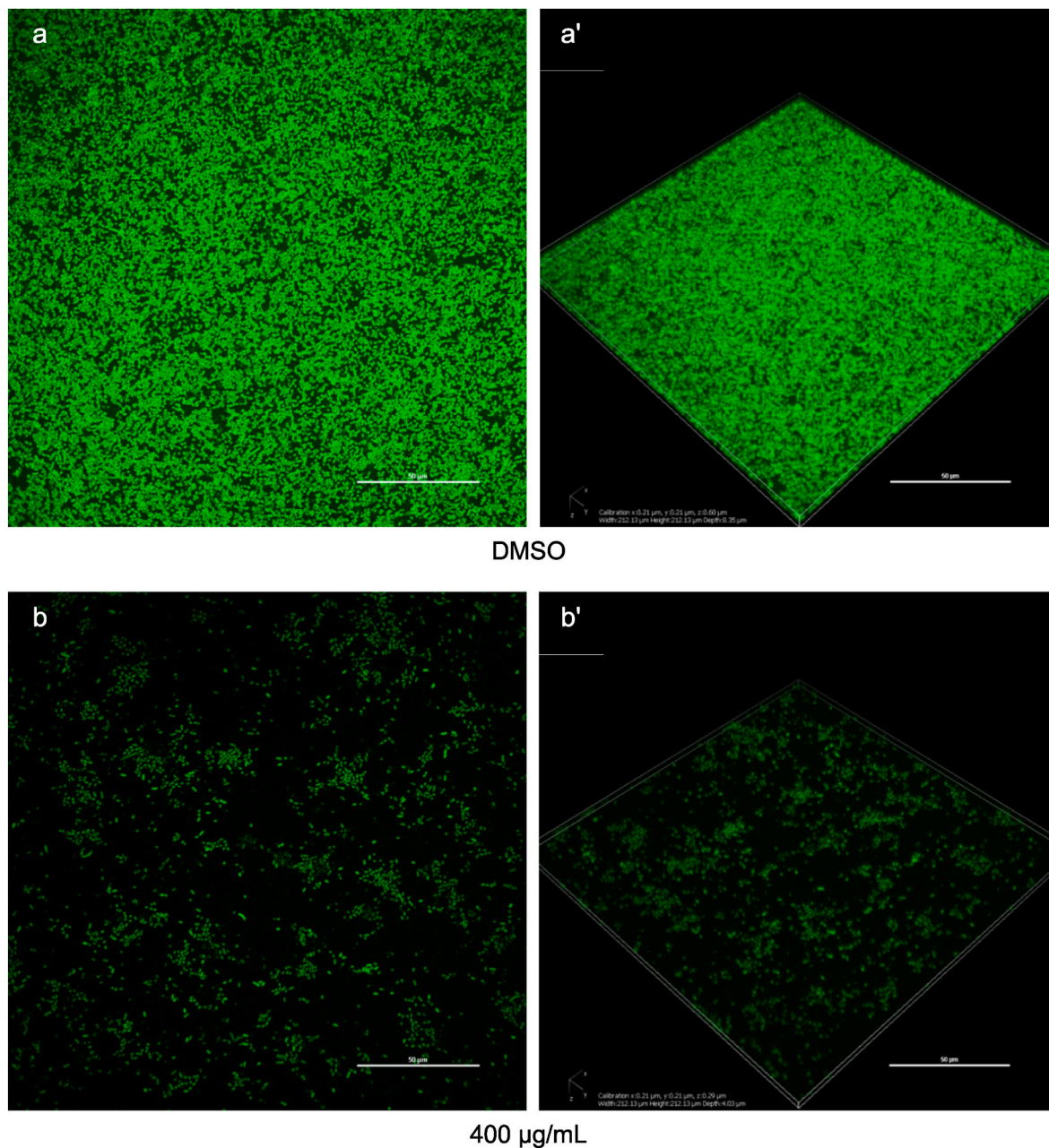
## 2.7. Expression profile of virulence genes in *S. marcescens* NJ01

The alteration of gene expression profile associated with virulence factors in *S. marcescens* NJ01 was analyzed by RT-qPCR. The expression of QS-related virulence genes *flhD*, *fimA* and *fimC* were significantly downregulated by 31%, 47% and 6%, respectively, when treated with 400 µg/mL of *C. sphaerospermum* extracts. Further, the virulence genes *bsmA* (15%) and *bsmB* (27%) exhibited downregulated gene expression. A downregulation in gene expression was also observed for the virulence genes *pigA* (30%), *pigC* (33%) and *shlA* (10%) (Fig. 11).

## 3. Materials and methods

### 3.1. Strains

The deep-sea-derived fungus SCSGAF0054 was obtained from the



**Fig. 7.** Confocal laser scanning microscopic observation of *S. marcescens* NJ01 biofilms treated with *C. sphaerospermum* extracts (a) and (a') DMSO (b) and (b') 400 µg/mL extracts.

Third Institute of Oceanography (TIO) of the Ministry of Natural Resources. *S. marcescens* NJ01 was acquired from Q.H. Gong (Ocean University of China) which was cultivated in Luria-Bertani (LB) broth (Hopebio, Qingdao, China) and incubated at 28 °C, for 17 h at 180 rpm.

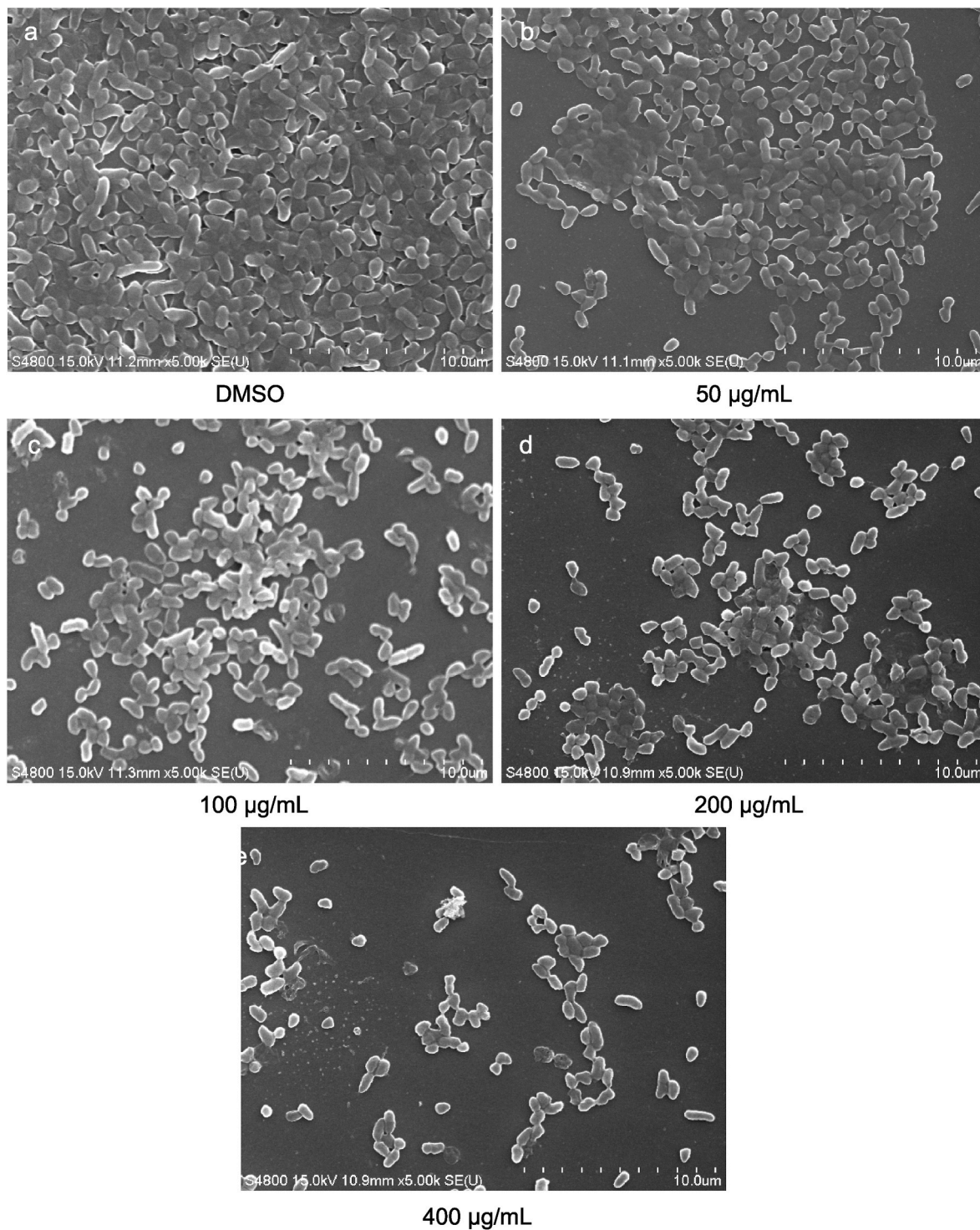
### 3.2. Fermentation and compound extraction from SCSGAF0054

The fungus SCSGAF0054 was cultivated at 28 °C on minimal glycerol (MG) plate for five days, then grown in FL3 liquid medium (5% mannitol, 0.75% yeast extract, 1.25% monosodium glutamate (MSG), 2.5% glucose, 0.75% maltose, 1.25% soya peptone) and incubated aerobically at 28 °C, 130 rpm for seven days. After fermentation, the solution was subjected to ultrasonic extraction using ethyl acetate. The extracts were obtained and stored for further experiments. The bacterial pathogen, *S. marcescens* NJ01 was cultivated in LB broth and maintained at 28 °C, 180 rpm for 17 h. Initially, 500 µL culture with  $OD_{620} = 0.1$  was

added to 50 mL of luke warm LB agar and mixed well. The mixture was poured into each petri plate and left for solidification. Then 30 µL extract was added to the solidified medium and incubated at 28 °C for 24 h. The anti-QS activity was assessed by observing the pigment inhibition zone.

### 3.3. LC-MS analysis of the extracts

The compounds were identified from the extracts of SCSGAF0054 by non-targeted LC-MS (Thermo Scientific™ Q Exactive™) analysis in the ion trap scanning mode. The mobile phase were prepared with sterilized distilled water with 0.1% of formic acid (solvent A) and HPLC grade 100% methanol (solvent B) [10]. The elution gradient program was carried out as follows, initially 98% A and 2% B for 1 min; 80% A and 20% B for 5 min; 50% A and 50% B for 10 min; 20% A and 80% B for 15 min; 5% A and 95% B for 20 min; 5% A and 95% B for 27 min; 98% A and 2% B for 28 min; 98% A and 2% B for 30 min. The sample injection

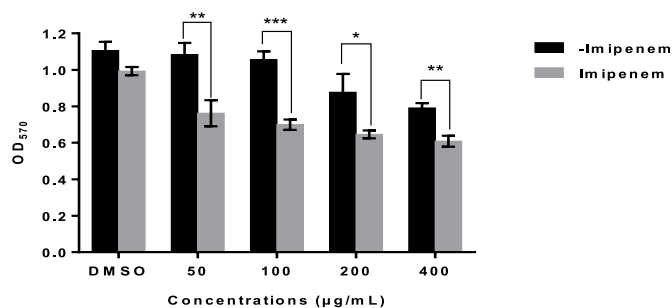


**Fig. 8.** Scanning electron microscopic observation of *S. marcescens* NJ01 biofilms treated with *C. sphaerospermum* extracts. (a) DMSO (b) 50 µg/mL extracts (c) 100 µg/mL extracts (d) 200 µg/mL extracts and (e) 400 µg/mL extracts.

volume was fixed at 2 µL. The obtained data were processed and analyzed using the Thermo Scientific Compound Discoverer 3.3 SP1 software. The detection of metabolites is primarily dependent on comparing MS/MS data to the mzCloud database. The unidentified signals were re-entered into the ChemSpider database using MS data and were confirmed by a FISH score greater than 50.

#### 3.4. MICs and growth measurement

The minimum inhibitory concentrations (MICs) of the extracts and imipenem against *S. marcescens* NJ01 were determined using CLSI method (Clinical and Laboratory Standards Institute, 2021). The pathogen *S. marcescens* NJ01 was cultivated and calibrated to 0.5 McFarland standard ( $1 \times 10^8$  CFU/mL). Different doses of active extracts (50–800 µg/mL) and were loaded into the each well contained *S. marcescens* NJ01 and incubated at 28 °C, 180 rpm for 24 h. The absorbance of the



**Fig. 9.** Dispersion of preformed biofilm by extracts combined with imipenem by crystal violet method. Data shown are mean  $\pm$  standard deviation ( $n = 3$ ).  $T$ -test was used to assess statistical significance. \* $p < 0.05$ , \*\* $p < 0.01$  and \*\*\* $p < 0.001$ .

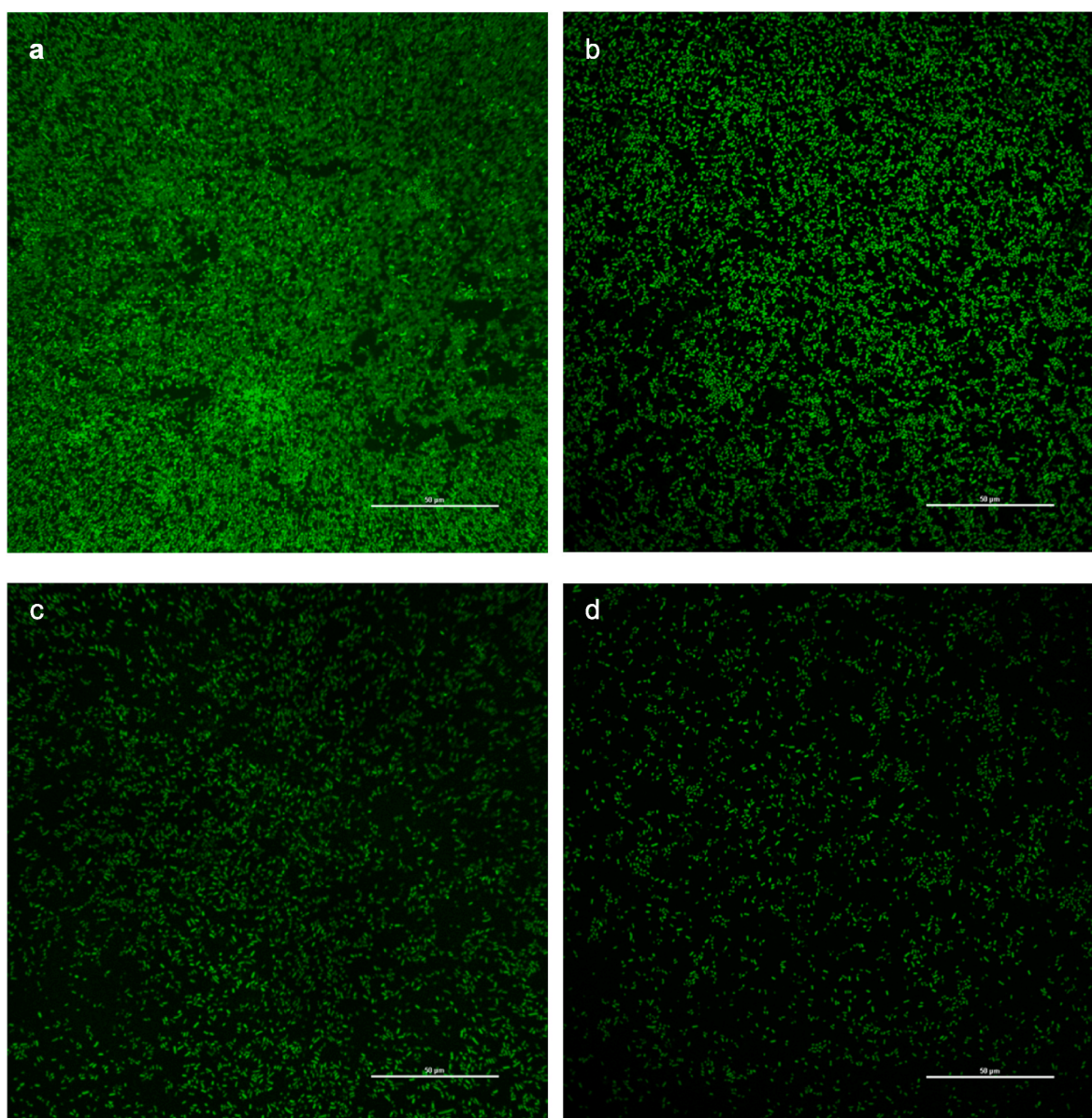
bacterial density was measured using 96-well micro plate reader at 620 nm [11].

The bacterial growth curve was measured by a slightly modified method [12]. The different concentrations of active extracts and the same amount of DMSO were added to the 0.5 McFarland calibrated cultures. The bacterial growth was measured at 620 nm every 2 h using a microplate reader.

### 3.5. Virulence factors assay

*S. marcescens* NJ01 was cultured in LB broth containing extracts at the desired concentrations and incubated for 24 h at 28 °C. DMSO and vanillic acid were served as the negative and positive controls, respectively. Further, the cultures were centrifuged at 15 294 $\times$ g for 5 min. The sterile supernatants and pellets were collected and used for the following studies [13].

For the determination of protease activity, 75  $\mu$ L of sterile supernatants were mixed with 125  $\mu$ L of 0.3% azocasein in 0.05 M Tris-HCl. Then the mixture was incubated at 37 °C for 3 h. The reaction was



**Fig. 10.** CLSM observation confirms that the synergistic effect of extracts and imipenem disintegrates preformed biofilm architecture. (a) DMSO (b) imipenem (c) 400  $\mu$ g/mL extract and (d) imipenem with 400  $\mu$ g/mL extract.

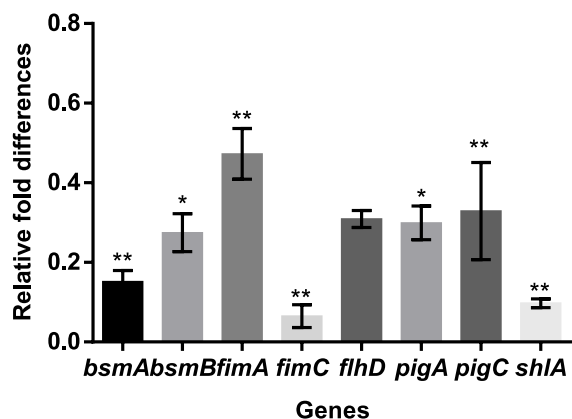


Fig. 11. Effects of the *C. sphaerospermum* extracts at 400 µg/mL on gene expressions of *S. marcescens* NJ01. Data shown are mean  $\pm$  standard deviation ( $n = 3$ ). T-test was used to assess statistical significance. \* $p < 0.05$ , \*\* $p < 0.01$ .

terminated by adding 600 µL of 10% trichloroacetic acid. After that, the reaction mixture was centrifuged at 15 294 $\times$ g at 4 °C and harvested supernatants. The absorbance of the mixture of 0.5 mL supernatants and equal volume sodium hydroxide solution was measured at 420 nm [14].

Prodigiosin production assay was performed based on previous report with minor modifications [15]. Briefly, 1 mL of acidified ethanol (4%, 1 M HCl) was mixed with the pellets. The solution mixture was centrifuged at 15 294 $\times$ g for 5 min at 4 °C and collected supernatants. The absorbance of the harvested supernatants was determined at OD<sub>534</sub>.

Lipase activity assay was performed based on previous report with minor modifications [16]. That is, 100 µL of cell-free supernatant was mixed with 900 µL of buffer solution (1 vol 0.3% (w/v) p-*p*-nitrobenzyl palmitate dissolved in isopropyl alcohol, 9 vol 0.2% (w/v) sodium deoxycholate and 0.1% (w/v) acacia dissolved in 50 mM disodium hydrogen phosphate) and incubated for 60 min. The solution mixture was centrifuged at 15 294 $\times$ g for 5 min and collected supernatants. The absorbance of the supernatants were measured at OD<sub>410</sub> and the amount of lipase was calculated.

The hemolysis production assay was performed based on previous report with minor modifications [17]. Briefly, 100 µL of the sterile supernatant was added with 900 µL of 2% sheep blood and incubated for 1 h. The mixture was centrifuged at 1 057 $\times$ g for 10 min. The absorbance of the harvested supernatants was measured at 530 nm and the hemolysis production was calculated.

The exopolysaccharides (EPS) production assay was performed based on previous report with minor modifications [18]. That is, 1 mL of 0.4 M sodium hydroxide solution was added to the pellet and dissolved by shaking for 15 min. The supernatant was collected after 15 min of centrifugation at 1 057 $\times$ g. The insoluble EPS was obtained by filtering the above supernatant. The EPS was precipitated by adding two volumes of absolute ice-cold ethanol to the supernatant and kept at 4 °C for 24 h. The crude EPS precipitate was then recovered by centrifugation at 12 000 $\times$ g for 20 min at 4 °C and dissolved in distilled water. The EPS was lyophilized and dissolved in 125 µL of 0.4 M sodium hydroxide solution. Then, 500 µL sulphuric acid was added and mixed thoroughly. Subsequently, 100 µL of 5% phenol was added to the solution mixture and incubated at 37 °C for 15 min. Finally, the EPS content was evaluated at 490 nm using microplate reader.

### 3.6. Swimming assay

The swimming assay was performed based on previous report with minor modifications [19]. The swimming motility was evaluated on LB agar plates (0.3% Agar, 0.5% NaCl, 1% peptone, pH 7.2) modified with

the desired concentration of bioactive extracts. *S. marcescens* NJ01 was spotted on the center of the plate using a sterile swab and incubated at 28 °C for 24 h in an upright position. DMSO served as the control group. In the plates, the swimming motility of the cells was observed and the percentage of motility was estimated.

### 3.7. Biofilm inhibition assay

The biofilm inhibition assay was performed using a 96-well polystyrene microtitre plate [20]. Briefly, overnight broth culture of *S. marcescens* NJ01 was added to the freshly prepared LB broth containing the extracts (50–400 µg/mL) and incubated for 24 h at 28 °C. The biofilm was stabilized by adding 200 µL of methanol to each well for 15 min. Then the plate was desiccated at 60 °C. Biofilms were observed by labeling each well with 0.05% crystal violet dye (100 µL) after the plate was incubated at room temperature for 15 min. The biofilms were rinsed with phosphate-buffered saline (PBS) to remove planktonic cells and residual dye. Crystal violet was extracted from biofilms with 95% ethanol, and the absorbance of the dissolved pigment was monitored at 570 nm by employing a 96-well microtiter plate analyzer. The MTT test was employed to determine the number of colonies in biofilms.

### 3.8. CLSM and SEM analysis

Based on previous report with minor modifications [21]. Briefly, 1% overnight culture was added to LB broth with or without desired concentrations of extracts and incubated at 28 °C for 24 h. The planktonic bacteria on the coverslips were washed with PBS (pH 7.2).

The biofilms were stained with 0.1 mg/mL acridine orange (AO) and kept for 15 min. After that, the biofilms were rinsed thrice with PBS to remove residual dyes. Finally, the biofilms were fixed in coverslips and observed under confocal laser scanning microscopy (CLSM, Leica Microsystems, Germany).

The biofilms were fixed with 2.5% glutaraldehyde for 12 h of incubation. After drying the samples were dehydrated in a gradient of 50%, 70%, 80%, 90%, and 100% ethanol. The dried biofilm was sputtered with a two-sided metal stub of 10 nm gold (Au) thickness. The biofilm architecture and associated bacterial cell damage were observed by scanning electron microscopy (SEM, Thermo Scientific, Verios G4 UC, USA).

### 3.9. Dispersion of preformed biofilm by *C. sphaerospermum* extracts combining with imipenem

The dispersion of preformed biofilm by *C. sphaerospermum* extracts combining with imipenem were tested by the crystal violet method reported previously [20].

The bacterial biofilms were cultivated in LB broth at 28 °C for 24 h. Then the biofilms were washed with PBS (pH 7.2) to remove planktonic cells. Various concentrations of the extracts and imipenem were added to LB broth in a 96-well microplate containing biofilms, respectively. The plates were incubated at 28 °C for 24 h. After that, the biofilms were rinsed with PBS and subsequently 200 µL of methanol was added to each well to stabilize the biofilms for 15 min. Then the plate was dried at 60 °C. Biofilms were observed by staining each well with 0.05% crystal violet dye (100 µL) after the plate was incubated at room temperature for 15 min. The biofilms were rinsed with phosphate-buffered saline (PBS) to remove planktonic cells and residual dye. Crystal violet was eluted from biofilms with 95% ethanol, and the absorbance of the dissolved dye was evaluated at 570 nm by using a 96-well microtiter plate reader.

The biofilms were stained with AO (0.1 mg/mL) for 15 min. The residual stains were washed with PBS (pH 7.2). The coverslips were attached to the cavity slide and observed under CLSM (Leica Microsystem, Germany).



### 3.10. Expression profile of virulence genes by RT-qPCR analysis

The gene expression was performed by RT-PCR method with slight modifications [17]. The overnight culture was inoculated in LB broth containing 400 µg/mL of extracts and the same amount of DMSO and incubated for 24 h at 28 °C at 180 rpm. Subsequently, the cultured solution was centrifuged at 10 621×g for 10 min. Further, the total RNA was isolated from the bacterial pellets using Spin Column Bacteria Total RNA Purification kit (Solarbio Technology, Beijing, China). The cDNA synthesis was performed using Reverse Transcription Kit (with dsDNase) (LABGIC Technology, Beijing, China) synthesized cDNA for RT-qPCR detection. Supreme Enzyme Mix (LABGIC Technology, Beijing, China), including Reverse Transcriptase, Ribonuclease Inhibitor and dsDNase, was used to remove genomic DNA and reverse transcription. RT-qPCR was carried out using SYBR Green qPCR Mix Kit (LABGIC Technology, Beijing, China) on a fluorescence quantitative PCR instrument (Analytik-qTOEWER3). The housekeeping gene *rpsL* was used as a reference gene. The primer details of genes are shown in Table S1.

### 3.11. Statistical analysis

All experiments were performed in triplicate. Statistical differences were evaluated by one-way analysis of variance (ANOVA) or *t*-test using GraphPad Prism 6 (GraphPad Software Inc., USA). Statistical significance was expressed using the following terminologies: \**p* < 0.05, \*\**p* < 0.01, \*\*\**p* < 0.001.

## 4. Discussion

*Serratia marcescens* NJ01 has an innate resistance to various antimicrobial agents, such as antibiotics and antimicrobial peptides [3]. Clinical isolates of *S. marcescens* are frequently identified as multidrug-resistant [22]. The suppression of quorum sensing is an alternative to hindering bacterial growth to mitigate bacterial toxicity and treating infections [23]. Quorum-sensing inhibitors (QSIs) have been demonstrated to reduce virulence and increase antibiotic susceptibility. Microorganisms originating from the ocean's depths are more likely to produce new bioactive molecules with unique mechanisms of action for drug discovery [24]. *C. sphaerospermum* from the deep sea has produced an abundance of bioactive molecules such as macrolides, alkaloids, tetramic acid derivatives etc [8,25]. The secondary metabolites of the deep-sea fungus *C. sphaerospermum* EIODSF 008 exhibited considerable antibacterial effects in preliminary studies [26]. Cladonion was isolated from *C. sphaerospermum* Z148, showing QSI activity against *P. aeruginosa* [9]. The marine-derived fungus *Cladosporium* sp. KFD33 was isolated from a blood cockle in China's Haikou Bay. Altertoxins VIII–XII and cladosporol I were extracted from this fungus fermentation liquid and have been used as a QSI against *C. violaceum* CV026 [27]. However, no reports of *C. sphaerospermum* against *S. marcescens* NJ01 have been found. Hence, the present investigation aims to evaluate the inhibitory effects of *C. sphaerospermum* extracts SCGAF0054 against the QS-associated virulence factors and biofilm formation of *S. marcescens* NJ01.

The SCGAF0054 extracts contained 1 057 compounds that were identified in the mzCloud database. Among these, a total of 498 compounds exhibited a strong correlation with the mzCloud database, with a score greater than 60. In LC-MS spectra, the compound with a Rt of 4.7 min was identified as thymine which has been reported to increase the sensitivity of numerous gram-negative pathogens to multiple antibiotics by activating bacterial metabolism and respiration, triggering the generation of reactive oxygen species (ROS) [28]. The compound with a Rt of 1.5 min was pyridoxal, a vitamin B6 that served an essential role in maintaining epidermal function. Further, the compound with a Rt of 12.1 min was cyclo(L-Phe-L-Pro). Previous reports suggested that the Cyclo(L-Phe-L-Pro) (cyclic dipeptide) isolated from the marine fungus *C. sphaerospermum* F14. It has been reported to have numerous

biological activities, including anti-inflammatory, antitumor, and anti-bacterial properties against *L. hongkongensis*, *M. luteus* and *Ruegeria* sp [29,30]. The compound with a Rt of 11.5 min was 3-phenyllactic acid which exhibited QSI and anti-biofilm activity against *P. aeruginosa* [21, 31]. Anthranilic acid with a Rt of 10.0 min, could inhibit hypha formation in *S. scitamineum* [32,33]. Benzoic acid with a Rt of 10.1 min, which showed QSI and anti-biofilm activity against *E. coli* K88 [34]. Some of these compounds have been reported to possess QSI or anti-biofilm activity. The extract of SCGAF0054 exhibited significant QSI and anti-biofilm activity against *S. marcescens* NJ01. However, these compounds are not the main ingredients of the extracts. Therefore, we hypothesized that all the compounds possessed QSI and anti-biofilm activity against *S. marcescens* NJ01. In addition, *C. sphaerospermum* contains multiple secondary metabolites and hence it was hypothesized that the extracts could contain novel anti-quorum sensing and anti-biofilm compounds.

Numerous virulence factors of *S. marcescens*, including biofilm formation, motility, prodigiosin, protease, lipase and hemolysin, are associated with QS [11,35]. Protease in *S. marcescens* can degrade immunoglobulins A and G in the host, promoting bacterial invasion and inhibiting host defense [36,37]. The cytotoxicity of hemolysin damages host cells through inflammation and hinders neutrophils defensive function [38]. The chosen concentrations of *C. sphaerospermum* extracts were selected below the MIC value against *S. marcescens*. *S. marcescens* NJ01 treated with 400 µg/mL SCGAF0054 extracts showed reductions in the synthesis of prodigiosin (57%), protease (46%), lipase (37%), hemolysin (37%), and EPS (50%). The motility of *S. marcescens* NJ01 was reduced when treated with various concentrations of extracts. Swimming mediated by flagella was decreased by 53% at 400 µg/mL. In a previous study, 3-phenylpropan-1-amine (3-PPA) was found to inhibit *S. marcescens* NJ01 biofilm formation and virulence factors, such as prodigiosin, protease, lipase, hemolysin, and swimming motility [13].

*S. marcescens* NJ01 forms biofilms through a series of defined stages that culminate in a highly porous, filamentous biofilm composed of cell chains, filaments, and cell clusters dependent on the quorum-sensing systems [13,39,40]. Bacteria susceptible to antimicrobial agents can become resistant after forming a biofilm. According to reports, biofilm is a major contributor to bacterial resistance and chronic infection [16, 28]. *S. marcescens* NJ01 was treated with 400 µg/mL extracts of SCGAF0054, which resulted in a reduction of biofilm formation (50%) and bacterial cell viability (44%). The results of SEM and CLSM indicated that biofilms were diminished, loosened and weakened after treatment with different concentrations of extracts, compared with control biofilms.

*S. marcescens* possesses innate resistance to numerous antibiotics, including carbapenems. In healthcare contexts, carbapenem-resistant *S. marcescens* (CRSM) is an emerging problem, and its enzyme can hydrolyze antibacterial drugs, including imipenem [3,41]. Synergistic effects of QS inhibitors and conventional antimicrobial drugs is a strategy to restore bacterial sensitivity [15,42]. In this study, SCGAF0054 extracts increased the susceptibility of *S. marcescens* NJ01 to imipenem and disrupted membrane structures.

Genes associated with virulence factors were selected to investigate the potential molecular mechanism of the extracts inhibiting virulence factors of *S. marcescens* NJ01. Bacterial fimbriae are crucial for colonization to human epithelial cells and biofilm formation [43]. It was demonstrated that type 1 fimbria of *S. marcescens* is encoded by *fimABCD* genes in the early stages of biofilm formation [44,45]. The extracts of SCGAF0054 inhibited the expression of *fimA* and *fimC* by 47% and 6%, respectively. The *flhD* gene was inhibited by 31%, which is responsible for bacterial motility. The biofilm formation genes including *bsmA* and *bsmB* were arrested by 15% and 27%, respectively. The extracts suppressed the prodigiosin-producing *pigA* and *pigC* genes expression by 30% and 33%, respectively. Moreover, the extracts inhibited the QS-associated hemolysin-encoding *shlA* (10%) gene expression.

## 5. Conclusion

This is the first study using the deep sea fungus, *C. sphaerospermum* extracts against *S. marcescens* pathogen. A total of 1 057 bioactive compounds were identified in the *C. sphaerospermum* extracts by LC-MS analysis. Among them, 498 compounds strongly correlated with the mzCloud database, with a score greater than 60. The biofilm formation and virulence factors of the *S. marcescens* NJ01, including prodigiosin, protease, lipase, hemolysin, EPS, and swimming motility, were considerably inhibited by various concentrations of *C. sphaerospermum* extracts. The SEM and CLSM images revealed that *C. sphaerospermum* extracts moderately arrested biofilm formation and cell viability. The combination of *C. sphaerospermum* extract with imipenam moderately disrupted preformed biofilm. RT-qPCR analysis revealed that the *C. sphaerospermum* extract moderately hindered the QS-associated virulence genes. These findings suggest that SCSGAF0054 extracts effectively inhibit the QS system to treat *S. marcescens* infections alone or in combination with conventional antimicrobial agents. Therefore, developing QSI derived from *C. sphaerospermum* extract to treat infections caused by *S. marcescens* and reduce its resistance is strongly encouraged.

## CRediT authorship contribution statement

**Dan-Rui Liu:** Writing – original draft, Visualization, Formal analysis, Methodology, Conceptualization, Data curation, Validation. **Qing-Xiang Yan:** Resources, Validation, Investigation. **Zheng-Biao Zou:** Visualization. **Chun-Lan Xie:** Validation. **Xian-Wen Yang:** Methodology, Writing – review & editing, Funding acquisition, Resources. **Ai-Qun Jia:** Methodology, Writing – review & editing, Funding acquisition, Resources.

## Declaration of competing interest

The authors declare have no competing financial interests.

## Data availability

Data will be made available on request.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.biofm.2023.100146>.

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