



Sotolon is a natural virulence mitigating agent in *Serratia marcescens*

Hisham A. Abbas¹ · Reham M. Goda²

Received: 31 March 2020 / Revised: 25 August 2020 / Accepted: 12 September 2020 / Published online: 24 September 2020
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Abstract

Serratia marcescens is an emerging opportunistic bacterium that can cause healthcare-associated infections. The high rate of multidrug resistance and the ability to produce a set of virulence factors, by which it can produce infectious diseases makes it urgent to find an alternative approach to the treatment of such infections. Disarming of virulence by targeting of quorum sensing (QS) as the regulating mechanism of virulence is a promising approach that has no effect on bacterial growth that is considered a key factor in emergence of resistance. This study was designed to investigate the ability of sub-inhibitory concentrations (sub-MICs) of sotolon to attenuate virulence of a clinical isolate of *S. marcescens*. Sotolon at 25 and 50 µg/ml inhibited 35.2 and 47.5% of biofilm formation, respectively. The inhibition of swimming motility were 41.4 and 69.3%, while that of swarming motility were 77.6 and 86.8% at 25 and 50 µg/ml, respectively. Moreover, sotolon reduced prodigiosin production by 76.6 and 87.6% at concentrations of 25 and 50 µg/ml, respectively. Protease activity was reduced by 25 µg/ml of sotolon by 54.8% and was completely blocked at 50 µg/ml. The relative expression of genes regulating virulence factors decreased by 40% for *fimA*, 29% for *fimC*, 59% for *flhC*, 57% for *flhD*, 39% for *bsmB*, 37% for *rssB*, 49% for *rsmA*, 54% for *pigP*, and 62% for *shlA* gene in the presence of 50 µg/ml sotolon. In conclusion, sotolon is an anti-virulence agent that could be used for the treatment of *S.marcescens* hospital-acquired infections.

Keywords *Serratia marcescens* · Antibiotic resistance · Anti-virulence · Sotolon

Introduction

Serratia marcescens is a facultative anaerobic motile Gram-negative bacillus that belongs to the family Enterobacteriaceae. It is of great importance due to the fact of being an opportunistic human pathogen that can cause a variety of healthcare-associated infections (Kida et al. 2007). The infections include central line associated blood stream infections, pneumonia, endocarditis, urinary tract infections and osteomyelitis (Van Houdt et al. 2007; Redondo-Bravo et al. 2019). Moreover, it is considered a food-borne pathogen because of its ability to colonize the gastrointestinal tract (Cristina et al. 2019). Furthermore, the common multidrug

resistance nature of *S. marcescens* complicates the treatment of its infections (González-Juarbe et al. 2015).

Many virulence factors contribute to the ability of *S. marcescens* to cause infections such as several extracellular enzyme including proteases, nucleases, lipases and hemolysin, as well as their swimming and swarming motilities (Sethupathy et al. 2017). *S. marcescens* also produces a characteristic red pigment; prodigiosin (2-methyl-3-pentyl-6-methoxyprodigiosin) that exerts antibacterial, anticancer, antiprotozoal, and immunosuppressant activities (Yip et al. 2019).

Many Gram-negative pathogens employ a cell-to-cell communication system called quorum sensing (QS). QS is a cell density dependent regulatory system of gene expression by employing signaling molecules or autoinducers termed N-acyl-homoserine lactones (AHLs) (Boyle et al. 2015; Bucio-Cano et al. 2015; Buseti et al. 2015). The LuxI protein family is responsible for the synthesis of AHLs that bind to receptors of the LuxR protein family. Such binding regulates the expression of many genes responsible for bioluminescence, production of pigments or antibiotics and virulence factors secretion (Morohoshi et al. 2008; Abisado et al. 2018). QS

Communicated by Erko stackebrandt.

✉ Hisham A. Abbas
hishamabbas2008@gmail.com

¹ Department of Microbiology and Immunology, Faculty of Pharmacy, Zagazig University, Zagazig, Egypt

² Department of Microbiology and Biotechnology, Faculty of Pharmacy, Delta University for Science and Technology, Gamasa, Mansoura, Egypt

system enables bacteria to behave in a community-like manner to perform many physiological functions, amongst which are the migration to more suitable environments, biofilm formation, virulence factors secretion, bacteriocin and antibiotic production, bioluminescence and pigment production (Skandamis and Nychas 2012; Martins et al. 2014).

QS is an attractive target for inhibition to control the virulence and pathogenesis of bacteria (Krzyżek 2019). QS inhibition can be achieved either by interfering with autoinducers synthesis, secretion or degradation, as well as by interfering with their recognition by proteins receptor such as those of the LuxR type homologs. As a result, the control of QS can be of great importance in control of infections (Jiang et al. 2019). The advantage of QS inhibition strategy is the lack of targeting the bacterial growth and imposing selective pressure on cells that leads to development of resistance (Asfour 2018). Thus, QS inhibition can be a good strategy for the treatment for *S. marcescens* infections that avoids the emergence of resistant strains as in the case with antibiotics.

Many natural compounds were used as QS inhibitors. One of the first used QS inhibitors were the halogenated furanones produced by the red alga *Delisea pulchra*. These compounds were found to inhibit quorum sensing in a number of bacteria (Zhang and Li 2015). In *Serratia*, halogenated furanones could block the swarming motility in *S. liquefaciens* MG1 strain (Rasmussen et al. 2000).

Sotolon (3-hydroxy-4,5-dimethyl-2(5H)-furanone) is a lactone compound that has an extremely powerful aroma. It bears the typical smell of fenugreek or curry when used at high concentrations, while it has maple syrup, caramel, or burnt sugar odor at lower concentrations. Sotolon is the major aroma and flavor component of fenugreek seed and lovage (Blank and Schieberle 1993). Sotolon was first isolated in 1975 from the herb fenugreek (Rijkens and Boelens 1975). Its name came from *soto*- "raw sugar" in Japanese and *-olon* signifies that the molecule is an enol lactone because it was found to be responsible for the flavor of raw cane sugar. Sotolon can pass through the body relatively unchanged, and when sotolon-rich foods such as fenugreek are consumed by human, a maple syrup aroma can be imparted to one's sweat and urine (Yukiko et al. 1980).

In this work, the anti-virulence activities of sotolon as a furanone compound was investigated against a clinical isolate of *S. marcescens* from a patient with nosocomial pneumonia.

Materials and methods

Bacterial strain

A clinical isolate of *S. marcescens* recovered from a patient with nosocomial pneumonia at the intensive care unit at

Zagazig University Hospital by endotracheal aspiration and identified using the MALDI-TOFF apparatus at the Clinical Pathology Department, Faculty of Medicine, Zagazig University was used in this investigation (Abbas and Hegazy 2017).

Determination of minimum inhibitory concentration (MIC) of sotolon

The broth microdilution method was used to determine the minimum inhibitory concentration (MIC) of sotolon (CLSI 2012). *S. marcescens* was cultivated in Mueller–Hinton (MH) broth (Oxoid, Hampshire, UK) at 37 °C for 18 h. Then, this culture was diluted with MH broth to have a turbidity equivalent to 0.5 McFarland standard and, then diluted (1:100) to have a final cell density of approximately 10⁶ CFU/ml. Different dilutions of sotolon (400, 200, 100, 50, 25, 12.5 µg/ml) were prepared in MH broth, then aliquots of 100 µl aliquots of these dilutions were transferred into the wells of 96-well microtiter plate. To the wells containing sotolon dilutions, aliquots of 100 µl of the prepared *S. marcescens* suspension were added. The microtiter plate was incubated at 37 °C and also at 28 °C for 20 h and MIC of sotolon was calculated by determination of the lowest concentration of sotolon that showed no visible growth.

Sub-inhibitory concentrations (sub-MICs) of sotolon (50 and 25 µg/ml corresponding to 1/4 and 1/8 MIC values, respectively) were used to assess the effect of sotolon on virulence of *S. marcescens*.

Biofilm formation assay

Biofilm was allowed to form in the presence and absence of sub-MICs of sotolon (Abraham et al. 2011). *S. marcescens* was grown in tryptone soya broth (TSB; Oxoid, Hampshire, UK) for 18 h at 28 °C and TSB was used to dilute the culture to have a suspension with optical density at 600 nm (OD₆₀₀) of 0.4 measured using Biotek spectrofluorometer (Biotek, USA). Aliquots of 10 µl of this suspension were added to 1 ml of fresh TSB with sotolon and control TSB and 100 µl of TSB preparations were transferred into the wells of 96-well microtiter plate. The plate was incubated at 28 °C for 24 h and the planktonic cells were removed, then the wells were washed with distilled water and dried. The adherent cells were methanol-fixed for 20 min and stained with 1% crystal violet. After 20 min, the wells were washed and the attached stain was dissolved using glacial acetic acid (33%). The absorbance of treated and untreated samples was measured at 590 nm using

Biotek spectrofluorometer and the percentage of biofilm inhibition was calculated using the following formula:

$$\% \text{ of biofilm inhibition} = \left[\text{OD}_{590} \text{ control} - \text{OD}_{590} \text{ in presence of sotolon} \right] \times 100 / \text{OD}_{590} \text{ control.}$$

with and without sotolon that was incubated at 28 °C for 18 h. The suspensions were centrifuged at 10,000 rpm for

Swimming and swarming motilities assay

To detect the possible anti-swarming and anti-swimming activities of sotolon, sotolon-containing swimming Luria–Bertani (LB) agar (Lab M Limited, Lancashire, United Kingdom) plates (0.3% agar) and sotolon-containing swarming LB agar plates (0.5% agar) and control plates were prepared. Culture of *S. marcescens* in LB broth was prepared by culturing the bacteria at 28 °C for 18 h, from which 5 µl aliquots of were inoculated into the center of the swimming plates, and were point inoculated in the swarming plates. The plates were incubated at 28 °C for 20 h and both the swimming and swarming zones were measured (Matsuyama et al. 1992).

Prodigiosin production assay

Quantification of prodigiosin production by *S. marcescens* was performed in the presence and absence of sotolon. The bacterial strain was grown in LB broth for 16 h at 37 °C, diluted to have a suspension with OD₆₀₀ equal to 0.4 as measured by Biotek Spectrofluorometer and then inoculated in 2 ml fresh LB broth with and without sotolon. LB broth tubes were incubated at 28 °C for 18 h and then centrifuged at 13,000 rpm for 10 min to collect the cell pellets. Prodigiosin was extracted by acidified ethanol (4 ml of 1 M HCl in 96 ml of ethanol). and the tubes were centrifuged again to separate the supernatants. The absorbance of the supernatants was measured at 534 nm using Biotek Spectrofluorometer and the degree of prodigiosin inhibition was determined. The inhibition percentage was calculated as % of prodigiosin inhibition = [absorbance of control – absorbance in presence of sotolon] x 100/absorbance of control (Gowrishankar et al. 2014).

Proteolytic activity assay

To assess the inhibition of proteolytic activity of the tested *S. marcescens* by sotolon, the skim milk agar method was used. Wells were made in skim milk LB agar plates (5% skim milk). *S. marcescens* was grown in LB broth for 16 h at 37 °C and then diluted to have a suspension with OD₆₀₀ equal to 0.4 as measured by Biotek spectrofluorometer. The prepared suspension was used to inoculate fresh LB broth

15 min to separate the supernatants. Aliquots of 100 µl of the supernatants of treated and untreated samples were transferred to the wells and the plates were incubated for 18 h at 37 °C. The proteolytic activity was determined by measuring the clear zones around the wells. (Hassett et al. 1995).

Quantification of the expression of virulence genes by quantitative real-time qRT-PCR

For RNA extraction, *S. marcescens* was grown in LB broth with 50 µg/ml of sotolon and in control LB broth and incubated for 18 h at 28 °C. The suspensions were centrifuged at 10,000 rpm for 15 min to separate the pellets. The bacterial cells were lysed using RNA lysis buffer, then total RNA was isolated with RNeasy Mini Kit (Qiagen, Germany) and further analyzed for quantity and quality with the spectrophotometer (Beckman, USA).

Total RNA from each sample (10 ng) was used for cDNA synthesis by reverse transcription using High-capacity cDNA Reverse Transcriptase kit (Applied Biosystem, USA). Then, cDNA was amplified using the Syber Green I PCR Master Kit (Fermentas, USA) in a 48-well plate by employing the Step One instrument (Applied Biosystem, USA) as follows: enzyme activation (10 min at 95 °C), followed by amplification (40 cycles of 15 s at 95 °C, 20 s at 55–65 °C and 30 s at 72 °C). The 2^{-ΔΔC_t} method was used to measure the changes in the expression of each target gene that were normalized relative to the mean critical threshold (CT) values of the housekeeping *rplU* gene (Salini and Pandian 2015). Aliquots of 1 µM of both primers specific for each target gene were used. The sequence of the primers sequence and the annealing temperature specific for each gene are demonstrated in Table 1.

Statistical analysis

The experiments were made in triplicates except for the qRT-PCR of the virulence genes expression which was made in duplicate. One way ANOVA test, Graph Pad Prism 5 was used for statistical analysis of the inhibitory activities of sotolon on virulence factors of *S. marcescens*. *P* values < 0.05 were considered statistically significant.

Table 1 Sequences of the primers and annealing temperatures for virulence genes

Target gene	Sequence (5'–3')	Annealing temperature
<i>fimA</i>	Forward: ACTACACCCTGCGTTTCGAC	58 °C
	Reverse: GCGTTAGAGTTTGCCTGACC	
<i>fimC</i>	Forward: AAGATCGCACCGTACAAA CC	55 °C
	Reverse: TTTGCACGCATAGTTCAAG	
<i>flhC</i>	Forward: AAGAAGCCAAGGACATTC AG	60 °C
	Reverse: TTCCCAGGTCATAAACCAGT	
<i>flhD</i>	Forward: TGTCGGGATGGGGAATAT GG	55 °C
	Reverse: CGATAGCTCTTGCAGTAA ATGG	
<i>bsmB</i>	Forward: CCGCCTGCAAGAAAGAAC TT	62 °C
	Reverse: AGAGATCGACGGTCAGTTCC	
<i>rssB</i>	Forward: TAACGAAGCTGCTGATGCTGT	58 °C
	Reverse: GATCTTGCGCCGTAAATTAT	
<i>rsmA</i>	Forward: TTGGTCAAACCCTCATGATT	65 °C
	Reverse: GCTTCGGAATCAGTAAGTCG	
<i>pigP</i>	Forward: GAACATGTTGGCAATGAA AA	55 °C
	Reverse: ATGTAACCCAGGAATTGCAC	
<i>shlA</i>	Forward: GCGGCGATAACTATCAAAAAT	55 °C
	Reverse: ATTGCCAGGAGTAGAACCAG	
<i>rplU</i>	Forward: GCTTGAAAAGCTGGACA TC	65 °C
	Reverse: TACGGTGGTGTTTACGACGA	

Results

Antibacterial activity of sotolon

Sotolon inhibited the growth of *S. marcescens* at 200 µg/ml. The potential inhibitory effects of sotolon against virulence factors of *S. marcescens* was investigated at two sub-MICs; 25 and 50 µg/ml that are equivalent to 1/8 and 1/4 MIC values, respectively.

Sotolon reduced biofilm formation

In previous study on *S. marcescens* clinical isolate, it was found that it had a strong ability to form biofilm (Abbas and Hegazy 2017). Sotolon significantly reduced biofilm formation by *S. marcescens* ($p < 0.05$) by 35.2% at 25 µg/ml and 47.5% at 50 µg/ml (Fig. 1).

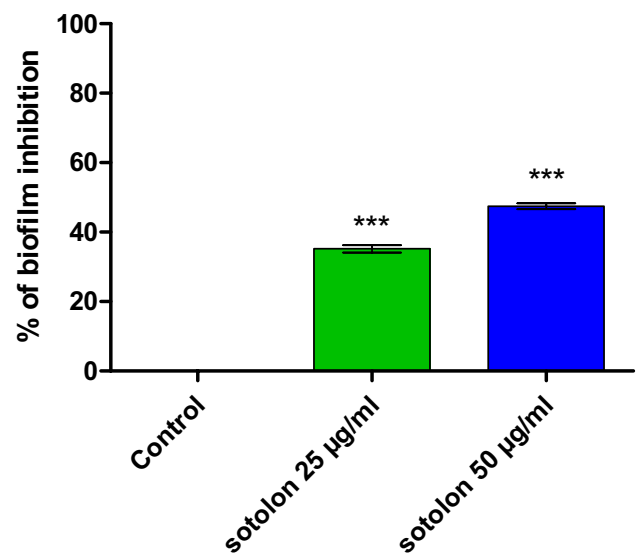


Fig. 1 Inhibition of biofilm formation of *S. marcescens* by different sub-inhibitory concentrations (sub-MICs) of sotolon in tryptone soya broth (TSB) at 28 °C for 24 h. *** statistically significant reduction of biofilm formation with sotolon

Sotolon inhibited swimming and swarming motilities

Swimming and swarming motility are related to the adhesion of bacteria to host tissues and the ability to form biofilms. The zones of swimming and swarming formed in swimming and swarming LB agar plates with and without sotolon were measured. At 25 µg/ml of sotolon, swimming motility was reduced by 41.4%, while swarming motility decreased by 77.6%. Moreover, swimming and swarming motilities were reduced by 69.3 and 86.8%, respectively, in the presence of 50 µg/ml of sotolon (Figs. 2 and 3).

Sotolon inhibited prodigiosin production

The intracellular red prodigiosin pigment is produced by *S. marcescens* and its production is under the control of the QS machinery. Prodigiosin inhibition by sotolon was found to be remarkable. The inhibition achieved ranged between 76.6 and 87.6% at concentrations of 25 and 50 µg/ml, respectively (Fig. 4).

Sotolon diminished protease activity

To investigate the possible inhibition of protease activity by sotolon, the skim milk agar method was used and the diameters of the clear zones surrounding the wells made in skim milk LB agar plates were determined. Protease activity was

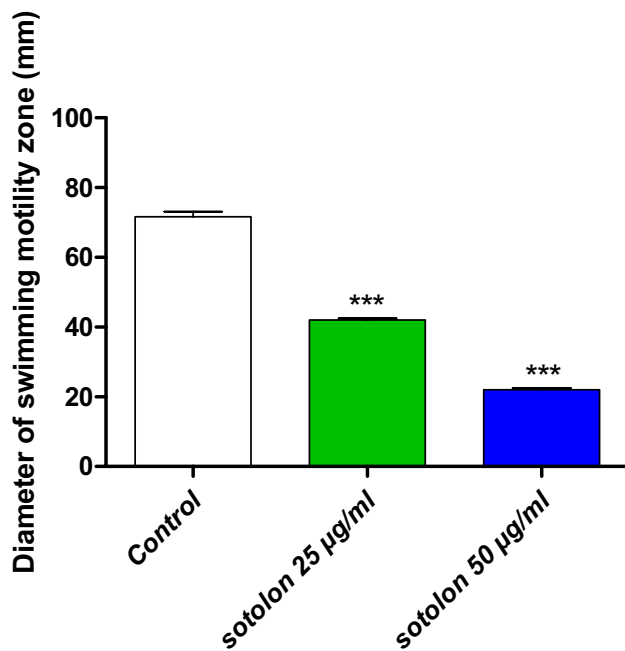


Fig. 2 Inhibition of swimming motility of *Serratia marcescens* by different sub-inhibitory concentrations (sub-MICs) of sotonol in Luria–Bertani (LB) agar plate (0.3% agar) at 28 °C for 20 h. *** statistically significant reduction of swimming motility with sotonol

reduced by 54.8% at 25 µg/ml of sotonol and was completely blocked at 50 µg/ml (Fig. 5).

Sotonol reduced the expression of virulence genes in *S. marcescens*

For confirmation of the ability of sotonol to inhibit the virulence factors in *S. marcescens* at the molecular level, qRT-PCR was used (Fig. 6). Interestingly, it was found that sotonol significantly reduced the levels of expression of all tested virulence genes as shown by comparing the expression levels in sotonol treated *S. marcescens* and in control *S. marcescens* by the $2^{-\Delta\Delta C_t}$ method. The relative expression of genes regulating virulence factors production decreased by 40% for *fimA*, 29% for *fimC*, 59% for *flhC*, 57% for *flhD*, 39% for *bsmB*, 37% for *rssB*, 49% for *rsmA*, 54% for *pigP*, and 62% for *shlA* gene.

Discussion

S. marcescens is one of the clinically significant bacteria because it is frequently involved in diverse healthcare-associated infections and it can also cause food infection (Van Houdt et al. 2007; Cristina et al. 2019). *S. marcescens* pathogenesis is complicated by its ability to secrete

several virulence factors such as proteases, lipases, nucleases and hemolysin, its swimming and swarming motilities and the emergence of multidrug resistance (Abbas and Hegazy 2020). The high rate of multidrug resistance necessitates extensive research to find new drugs to treat resistant bacterial infections. Several approaches are now investigated, among which the targeting of the intercellular communication system termed QS that regulates the production of virulence factors (Gebreyohannes et al. 2019; Mion et al. 2019).

In this study, the natural compound sotonol was investigated for its virulence attenuating activity.

Sotonol was tested as *S. marcescens* virulence attenuating agent at sub-MICs (25 µg/ml equivalent to 1/4 MIC and 50 µg/ml equivalent to 1/8 MIC) to avoid any possible bacterial growth inhibitory activity.

Biofilm formation represents a major cause of antibiotic resistance in *S. marcescens*. By adhesion to living surfaces or medical devices, *S. marcescens* can form biofilms resulting in life-threatening infections (Shanks et al. 2007; Satpathy et al. 2016). Sotonol was found to significantly inhibit biofilm formation. Moreover, the ability of *S. marcescens* to swim and to swarm that are linked to biofilm formation capability was blocked by sotonol. The swarming inhibiting activity of sotonol was more pronounced than its swimming inhibiting one.

S. marcescens strains were found to produce the red prodigiosin pigment (Lin et al. 2019). Prodigiosin production is directly regulated by QS. Sotonol could reduce prodigiosin production to a significant extent. This indicates the possible anti-QS activity of sotonol.

S. marcescens can modulate the immunity of the host by secretion of extracellular proteases (Sethupathy et al. 2016). Protease activity in the presence of 25 µg/ml of sotonol decreased by 54.8% and was completely abolished at 50 µg/ml.

Further confirmation of the anti-virulence activity of sotonol was performed at the molecular level by the qRT-PCR analysis. When the effect of sotonol on the expression of *fimA*, *fimC*, *flhC*, *flhD*, *rssB* and *rsmA* genes was performed, it was found that sotonol remarkably downregulated these genes that are involved in adhesion and swimming and swarming motilities. In *S. marcescens*, *fimA* and *fimC* are the major fimbrial subunits. The knock out of the *fimA* gene produced a mutant strain that lost the ability to form fimbriae (Labbate et al. 2007). Moreover, swimming and swarming motilities in addition to cell division and differentiation are regulated by the global gene regulators *flhD* and *flhC* that are produced under the control of the *flhDC* master operon. Furthermore, the *flhDC* promoter is regulated by the RssAB two compartment system (Srinivasan et al. 2017).

Sotonol also decreased the expression level of *bsmB* gene in *S. marcescens* clinical isolate. Mutation in *bsmB* resulted

Fig. 3 Inhibition of swarming motility of *S. marcescens* by different sub-inhibitory concentrations (sub-MICs) of sotolon in Luria–Bertani (LB) agar plate (0.5% agar) at 28 °C for 20 h. *** statistically significant reduction of swarming motility with sotolon

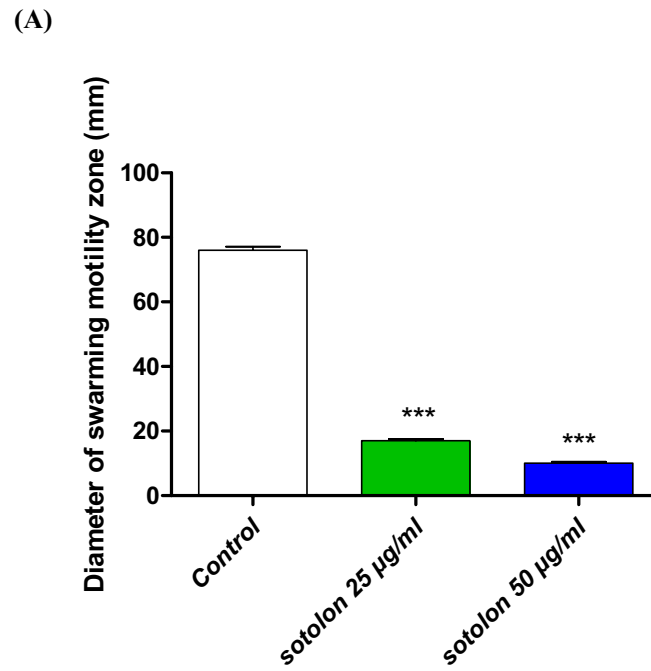


Fig. 4 Inhibition of prodigiosin production in *S. marcescens* by different sub-inhibitory concentrations (sub-MICs) of sotolon in Luria–Bertani (LB) broth at 28 °C for 18 h. *** statistically significant reduction of prodigiosin production with sotolon

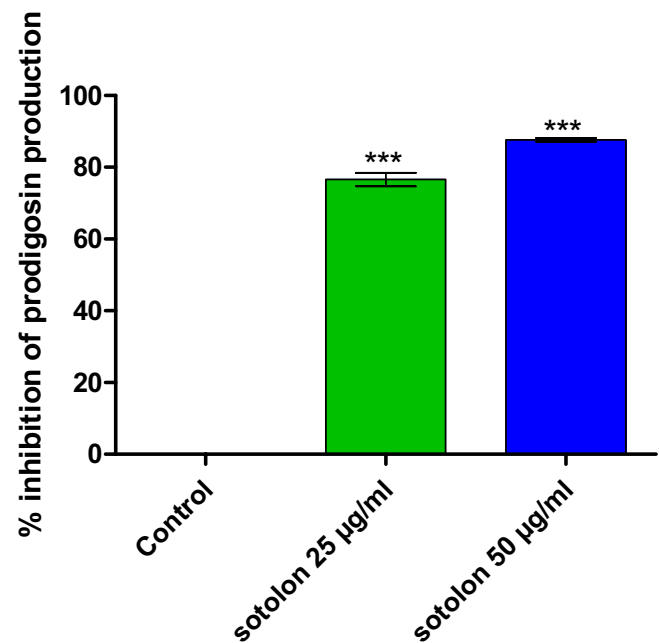


Fig. 5 Inhibition of protease activity of *S. marcescens* by different sub-inhibitory concentrations (sub-MICs) of sotolon in skim milk Luria–Bertani (LB) agar at 37 °C for 18 h. *** statistically significant reduction of protease activity with sotolon

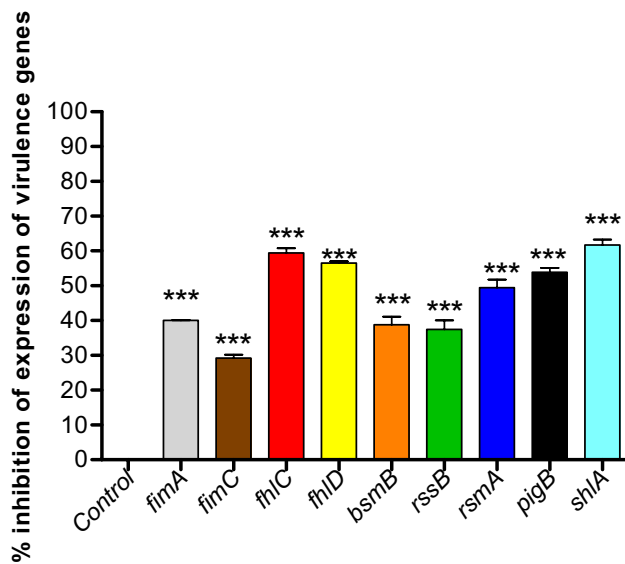
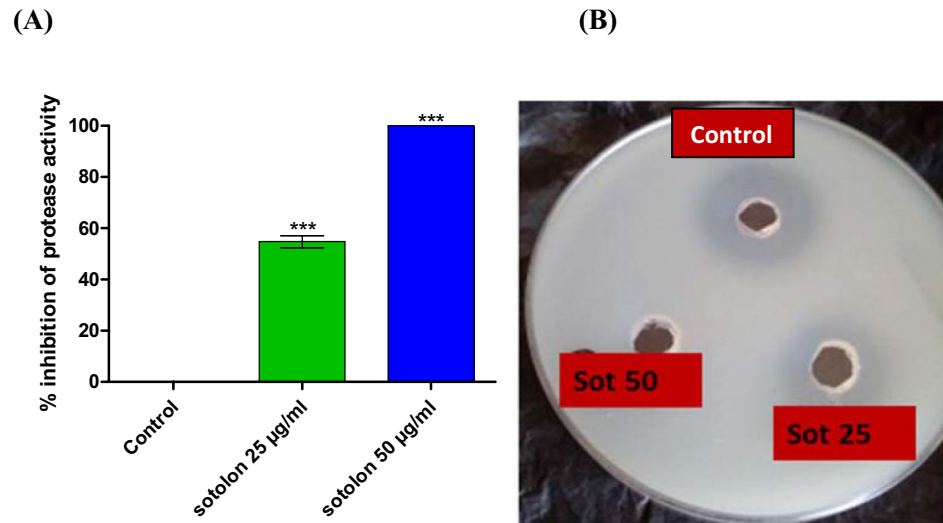


Fig. 6 Inhibition of the expression of virulence genes of *S. marcescens* by sub-inhibitory concentration (sub-MIC) of 50 µg/ml of sotolon. *** statistically significant decrease in the expression levels of virulence gene

in the inability of the mutant to form biofilm or to secrete lipase and protease (Labbate et al. 2007).

Sotolon reduced the level of the expression of *pigP* gene. The master transcriptional regulator *pigP* is responsible for the regulation of production of the QS-controlled *S. marcescens* prodigiosin pigment (Gristwood et al. 2011).

Sotolon-treated cells showed reduced expression of *shlA* gene. The major virulence gene *shlA* that was found to exert cytotoxic action against fibroblasts and epithelial cells (Di Venanzio et al. 2014). Mutants of *shlA* gene showed a significant decrease in the virulence of *S. marcescens* in mice (González-Juarbe et al. 2015).

Impeding QS by natural compounds is a promising approach for treating infections caused by *S. marcescens*. Examples of natural agents that could successfully interfere with QS are O-methyl ellagic acid and alpha-bisabolol (Salini and Pandian 2015; Sethupathy et al. 2016). QS inhibitors could also synergize antibiotics (Borges et al. 2014).

Sotolon is a furanone compound that has celery, nutty, spicy aroma. It is the major flavor compound of dried fenugreek seeds (Blank et al. 1997). The reason for selection of sotolon is its structural similarity to halogenated furanones produced by the marine algae *Delisea pulchra*. These furanones are quorum sensing inhibitors that were found to interfere with the algal surface colonization by other marine organisms (Dworjanyn et al. 2006). By mimicking the AHL signal through occupying the binding site on the putative regulatory protein, furanones make it highly unstable leading to fast disruption of the quorum sensing-mediated gene regulation (Eberl et al. 1999, Zhang and Li 2015). Also, synthetic furanones were found to block many bacterial quorum sensing and help decrease the severity of pulmonary infections (Wu et al. 2004). Ascorbic acid is a natural furanone that was found to block quorum sensing in *Pseudomonas aeruginosa* (El-Mowafy et al. 2014).

In conclusion, sotolon is a natural agent that could attenuate the virulence of *S. marcescens* and the mechanism of action can be the inhibition of QS. Thus, sotolon may be a promising anti-pathogenic natural agent to fight infections caused by *S. marcescens* in humans. To the best of our knowledge, this is the first study of sotolon against the virulence of *S. marcescens*.

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