

Research Paper

Anti-biofilm and Anti-Virulence Efficacy of Celastrol Against *Stenotrophomonas maltophilia*

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Abstract

Stenotrophomonas maltophilia is a multi-drug resistant opportunistic pathogen that causes nosocomial infections in immunocompromised patients. This pathogen is difficult to treat owing to its intrinsic multidrug resistance and ability to form antimicrobial-tolerant biofilms. In the present study, we aimed to assess the potential use of celastrol as a novel anti-biofilm and/or anti-virulence agent against *S. maltophilia*. Results showed that celastrol at its sub-inhibitory doses decreased biofilm formation and disrupt the established biofilms produced by *S. maltophilia*. Celastrol-induced decrease in biofilm formation was dose-dependent based on the results of the microtiter plate biofilm assays and confocal laser scanning microscopy. In addition, our data validated the anti-virulence efficacy of celastrol, wherein it significantly interfered with the production of protease and motility of *S. maltophilia*. To support these phenotypic results, transcriptional analysis revealed that celastrol down-regulated the expression of biofilm- and virulence- associated genes (*smeYZ*, *fsnR*, and *bfmAK*) in *S. maltophilia*. Interestingly, celastrol significantly inhibited the expression of *smeYZ* gene, which encodes the resistance-nodulation-division (RND)-type efflux pump, SmeYZ. Overall, our findings suggested that celastrol might be a promising bioactive agent for treatment of biofilm- and virulence-related infections caused by the multi-drug resistant *S. maltophilia*.

Key words: Celastrol, *Stenotrophomonas maltophilia*, Anti-biofilm, Anti-virulence, Resistance-nodulation-division (RND)-type efflux pump

Introduction

Stenotrophomonas maltophilia, a microorganism ubiquitously present in the environment, is an emerging multidrug resistant opportunistic human pathogen that often causes nosocomial infections. *S. maltophilia* is generally associated with septicemia and various infections, particularly respiratory infections, in immunosuppressed or immunocompromised patients, including those with cystic fibrosis (CF) [1, 2]. This aerobic gram-negative bacterium can also cause severe diseases, such as pneumonia, bacteremia, and endocarditis in patients exposed to invasive clinical devices and/or those receiving broad-spectrum antibiotics [3, 4]. One difficulty in treating *S. maltophilia* infections has been attributed to its intrinsic multidrug resistance [5]. Most clinical

isolates of *S. maltophilia* exhibit multidrug resistance to β -lactams, macrolides, and aminoglycosides owing to its multidrug efflux pumps and overexpression of resistant determinants, such as L1/L2 β -lactamases and aminoglycoside-modifying enzymes [3]. In addition, *S. maltophilia* genome encodes several molecular machineries that contribute to its resistance to various antibiotics [6]. Therefore, *S. maltophilia* has become a substantial threat to human health.

Resistance-nodulation-division (RND)-type efflux pumps can confer resistance against various drugs. They significantly contribute to the multidrug resistance of *S. maltophilia* [5]. Besides their known roles in antibiotic extrusion, RND-type efflux pumps are increasingly recognized as components required

for the bacterial physiological functions and virulence [7]. Of the diverse putative RND-type efflux systems described in *S. maltophilia*, SmeYZ efflux pump is constitutively expressed. It was shown that inactivation of *smeYZ* resulted in attenuation of drug resistance and virulence-associated characteristics, which decreased the *in vivo* virulence of *S. maltophilia* [8].

A broad spectrum of clinical syndromes has been shown to be caused by *S. maltophilia* infections [9]. However, little is known about its virulence factors. One of the most crucial virulence factors of *S. maltophilia* is its formidable ability to form biofilms, which are sessile communities of microbial cells that can adhere to one another on biotic and abiotic surfaces [1]. These adherent cells are often encased in a self-produced matrix of an extracellular polymeric substance, forming a complex three-dimensional architecture [10-12]. Biofilm-grown cells exhibit phenotypic properties that differ from those of the organisms grown planktonically, including increased resistance to multiple antibiotics, host immune responses, and multiple stressors [13-15]. One of the mechanisms that afford resistance against numerous antibiotics involve the delayed penetration of the antimicrobial drugs through the multilayer biofilm matrix. Therefore, treatment of biofilm-related infections is clinically difficult, resulting in chronic infections [16, 17].

Biofilm formation is a complex and multifactorial event. It depends on the surface characteristics, strain motility, genetic factors, and other factors [10, 12, 16]. Biofilm formation correlates with high resistance to antibiotics [18]. Recently, screening of a random transposon insertion mutant library of *S. maltophilia* ATCC 13637 identified the *fsnR* gene that encodes an orphan response regulator, FsnR. This regulatory protein plays a fundamental role in mediating the transcription of flagellar genes, cell motility, and biofilm formation [19]. Besides, two histidine kinase (HK) genes were shown to encode a two-component signal transduction system (TCS), the BfmAK system. This system has been confirmed to play a critical role in biofilm formation in *S. maltophilia* [20].

Conventional antibiotics used to treat biofilm-forming bacterial pathogens were not designed to act against the recalcitrant biofilm. Alternatively, they were designed to inhibit the growth of bacteria or kill them. This approach exerts selective pressure upon the pathogenic bacteria, which induces bacterial resistance against the particular drug being used [21]. With the increasing influence of drug resistance, novel therapies or antibiotic agents, including natural chemicals and

plant products that can regulate biofilm formation, remove established biofilms, and inhibit the expression of virulence-related factors, are needed to treat multidrug resistant *S. maltophilia* infections. These agents have received an increasing attention as a new class of antimicrobial compounds with anti-biofilm activity for treatment of infectious diseases [22, 23].

Celastrol is a pentacyclic triterpenoid compound mainly found in the roots of *Tripterygium wilfordii*, commonly known as thunder god vine. It has been shown that celastrol has multiple pharmacological effects, including anti-inflammatory, anti-cancer, hepatoprotective, and immunomodulatory effects [24, 25]. As a natural product, celastrol might exert inhibitory effects on biofilm formation. Recent *in vitro* studies showed that celastrol exhibited antimicrobial and anti-biofilm effects against the gram-positive pathogen *Staphylococcus aureus* [16, 26].

In this context, the present study was conducted with the aim of assessing the antimicrobial, anti-biofilm, and anti-virulence activities of celastrol against *S. maltophilia* using *in vitro* phenotypic and genotypic analyses.

Materials and Methods

Bacterial strains, growth measurements, and celastrol

S. maltophilia strain ATCC 13637 was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Clinical isolate, GNU2233 (from respiratory tract), were kindly provided by Gyeongsang National University Hospital Culture Collection for Pathogens. For general purpose, all strains were grown aerobically at 37 °C in cation-adjusted Mueller-Hinton broth (CA-MHB; Difco, Becton-Dickinson and Company, USA). For cell growth measurements, absorbance at 600 nm ($A_{600\text{ nm}}$) were measured using Multiskan GO plate reader (Thermo Fisher Scientific, USA). Each experiment was performed using at least three independent cultures, as described previously [26]. Celastrol was purchased from Cayman Chemicals (Ann Arbor, MI) and dissolved in dimethyl sulfoxide (DMSO).

Crystal violet biofilm assay

To determine the effect of celastrol on biofilm formation and established biofilms, crystal-violet staining experiments were performed [1]. First, *S. maltophilia* strains (ATCC 13637 and GNU2233) were inoculated into CA-MHB broth and cultured overnight to measure the blocking effect of celastrol on biofilm formation. *S. maltophilia* ATCC 13637 and GNU2233 were standardized to match approximately

0.5 McFarland standards. They were then diluted at 1:20 ($A_{600\text{ nm}}$, 0.005) with CA-MHB. Then 200 μL of the dilution was inoculated into the wells of sterile 96-well polystyrene tissue culture plate (Falcon) with or without sublethal celastrol concentrations (two-fold dilutions from 1.25 $\mu\text{g}/\text{mL}$ to 10 $\mu\text{g}/\text{mL}$ for ATCC 13637, and from 5 $\mu\text{g}/\text{mL}$ to 40 $\mu\text{g}/\text{mL}$ for GNU2233). For each concentration of celastrol, 8 wells were used. Media treated with 1% DMSO was used as positive control. After 24 h of incubation at 37 °C, the culture media was discarded and wells were washed three times with 200 μL of sterilized phosphate buffered saline (PBS) to remove non-adherent cells. Adherent biofilms were fixed by drying at 60 °C for 1 h and stained with Hucker-modified 0.1% crystal violet for 5 min. After discarding the remained stain by tapping on paper towel, stained biofilms were thoroughly rinsed three times with sterile deionized water, dried at 60 °C for 1 h, and extracted with 250 μL of 33% glacial acetic acid for 15 min, as described previously [26]. Dissolved biofilms were quantified by measuring the absorbance values at wavelength of 595 nm using the Multiskan GO plate reader. To examine the effect of celastrol on already established biofilms, 200 μL of bacteria culture diluted in media was inoculated into 96-well tissue culture plate and incubated at 37 °C for 24 h to allow biofilm formation. After the wells were washed three times with PBS, 200 μL of CA-MHB containing sub-inhibitory concentrations of celastrol was added to each well. After further incubation for 24 h, biofilms were stained and quantified as described above. Each experiment was performed at least three times in parallel.

XTT reduction assay

To measure cell viability of *S. maltophilia* in established biofilms following treatment with celastrol, XTT Cell proliferation kit (ATCC, Manassas, VA, USA) was used according to the manufacturer's recommendations [27]. As described above, biofilms of two *S. maltophilia* strains (ATCC 13637 and GNU2233) were prepared in 96-well polystyrene tissue culture plate. After 24 h of incubation, biofilms were rinsed three times with PBS and incubated with fresh CA-MHB media containing celastrol (10, 20, 40, and 80 $\mu\text{g}/\text{mL}$ for ATCC 13637 or 40, 60, 80, and 100 $\mu\text{g}/\text{mL}$ for GNU2233) or 1 % of DMSO for 24 h. Before reacting with cells from biofilms, XTT reagent and activation reagent were mixed at a ratio of 50:1 (v/v). Then 50 μL of activated-XTT solution was added into each well and incubated at 37 °C for 3 h in the dark. After incubation, reduction of XTT solution was determined by absorbance reading at wavelength of 475 nm using the Multiskan GO plate reader. The

absorbance reading at wavelength of 600 nm was used as background (to be subtracted from the reading at 475 nm).

Confocal laser scanning microscopy (CLSM)

Confocal laser scanning microscopy was performed to visualize biomass and cells of biofilm affected by celastrol using published procedures [28] with some modifications. Briefly, overnight bacterial culture of *S. maltophilia* ATCC 13637 was adjusted and inoculated into tissue culture treated 24-well glass bottom imaging plates (Effendorf AG, Hamburg, Germany, Cat. no.: 0030741021). After 24 h of incubation, suspension medium was discarded and each well was washed three times with 1 mL of sterile PBS. Preformed biofilms were then treated with celastrol (40 or 80 $\mu\text{g}/\text{mL}$) or DMSO 1 % (v/v) and incubated at 37 °C for 24 h. After washing out non-adherent cells with PBS, biofilms were fixed with 3.7 % (v/v) formaldehyde for 1 h, washed with sterile PBS, and then stained with three different fluorescent dyes. The amino group was stained with 10 $\mu\text{g}/\mu\text{L}$ of fluorescein isothiocyanate isomer I (FITC; Sigma-Aldrich, Germany) for 1 h at room temperature (RT). To visualize carbohydrate, wells were stained with 0.1 $\mu\text{g}/\mu\text{L}$ of Concanavalin A-Alexa Fluor 594 conjugate (ConA;C-11253, Molecular Probes, Eugene, OR, USA) at room temperature (RT) for 20 min. Extracellular nucleic acids were stained with 1 mg/L of 4, 6-diamidino-2-phenylindoldihydrochloride (DAPI; Molecular Probes, Eugene OR, USA) at RT for 40 min. After each staining step, wells were washed with 1 mL of PBS to remove unbound dye and dried for 15 min. All procedures were conducted in the dark. The excitation laser wavelengths for FITC, ConA, and DAPI were 495, 590, and 358 nm, respectively. Microscopic analysis was performed using a Zeiss LSM-710 confocal laser microscope (Carl Zeiss, Thornwood, NY, USA). Images were constructed using ZEN software (Carl Zeiss, Thornwood, NY, USA), as described previously [26].

Swimming motility assay

The effect of celastrol on swimming motility was assessed using 0.15% agar plate containing 1% tryptone and 0.5% NaCl as described previously [29] with slight modifications. Briefly, 1% DMSO (the control) or sublethal doses of celastrol was incorporated into motility agar plates. Two *S. maltophilia* strains (ATCC 13637 and GNU2233) grown overnight in CA-MHB under shaking conditions were diluted to OD_{600} of 1.0. Thereafter, 5 μL aliquot of bacterial culture was inoculated into the center of the motility agar surface. The inoculated plates were then incubated at 37 °C for 24 h and the diameter (mm) of

circular swimming zone was measured. Each experiment was performed in triplicates with three independent cultures.

Secreted protease activity assay

The effect of celastrol on the activity of protease secreted by two *S. maltophilia* strains (ATCC 13637 and GNU2233) was determined using Mueller-Hinton broth casein agar containing 1% skim milk as described previously [8] with some modifications. Briefly, each overnight culture grown in CA-MHB under shaking was adjusted to OD₆₀₀ of 0.005. The diluted bacterial culture was incubated with or without sublethal doses of celastrol at 37 °C for 24 h. Cell cultures were centrifuged at 12,000 × g for 15 min at 4 °C. Clear supernatant was collected from either celastrol treated or untreated sample via filtration using 0.45-μm filters (Corning). Thereafter, 40 μL of cell free culture supernatant (both treated and untreated) was inoculated into 6-mm diameter hole in the center of casein agar plate. After incubation at 37 °C for 48 h, proteolytic activities of cell free supernatants of strains were assessed by measuring the diameters of transparent zones surrounding the holes. Each experiment was performed in triplicates with three independent cultures.

RNA isolation

For transcriptional analysis of *S. maltophilia*, total bacterial RNA was isolated using the following procedure. Briefly, overnight cultures of *S. maltophilia* strains (ATCC 13637 and GNU2233) were diluted with 30 mL of CA-MHB medium to OD₆₀₀ of 0.005. All cultures were then grown at 37 °C for 6 h with shaking (250 rpm). After that, cell suspensions were cultured for another 4 hours at 37 °C in the presence or absence of celastrol at final concentration of 5 or 20 μg/mL. The two concentrations were chosen because they showed significant anti-biofilm and anti-virulence activities. Then 2 mL of bacterial culture from each sample was centrifuged at 25,000 × g for 1 min at 4 °C to collect cells. Bacterial total RNA was extracted and purified from pelleted cells using NucleoSpin RNA mini kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's protocol. To remove residual genomic DNA, extracted total RNA was filtrated with silica columns and subjected to DNase treatment. The quality and concentration of isolated RNA samples were assessed using BioDrop μLITE (BioDrop Ltd., Cambridge, UK), as described previously [26].

qRT-PCR

The transcription levels of virulence- and biofilm- related genes (*smeYZ*, *fsnR*, *bfmA*, and *bfmK*)

in *S. maltophilia* ATCC 13637 and GNU2233 treated with or without celastrol were determined using qRT-PCR. Gene-specific primers were used for the amplification of these genes. Transfer-messenger RNA (tmRNA) housekeeping gene [19] was used as an internal control. Primers used in this study were designed based on the genome sequence of *S. maltophilia* K279a [6]. Primer sequences are listed in Table 1. ReverTra Ace qPCR RT Master Mix with gDNA Remover (TOYOBO, Japan) was used to generate cDNA using 1 μg of total RNA. qRT-PCRs were performed in sets of triplicates using THUNDERBIRD SYBR qPCR Mix (TOYOBO, Japan) on an Mx3000P instrument (Agilent Technologies). Thermal cycling parameters were set as follows: at 95 °C for 1 min followed by 40 cycles of at 95 °C for 15 s and at 63 °C for *smeYZ* gene 1 min (at 60 °C for tmRNA gene for 1 min and at 61 °C for 1 min for other genes). This was followed by 1 cycle each at 95 °C for 1 min, at 60 °C for 30 s, and at 95 °C for 30 s. The expression values were normalized against tmRNA. All measurements were performed with three independent cultures and three replications within each qRT-PCR reaction, as described previously [26].

Table 1. Nucleotide sequences of primers used for quantitative RT-PCR analysis.

Genes	Primer sequences
<i>tmRNA</i>	Forward: 5'- GGG GGT GCA CTG GTT TCG -3' Reverse: 5'- TGG TGG AGG TGG GCG GAA T -3'
<i>smeYZ</i>	Forward: 5'- CAG ATC GAC CAG GTC TAC -3' Reverse: 5'- CAT TGA TAC CGG AGA ACA -3'
<i>fsnR</i>	Forward: 5'- CAT CCT TGA CCA CGA ACC -3' Reverse: 5'- TGA TGG ACC TGT CAT TGC -3'
<i>bfmA</i>	Forward: 5'- ATG ACG ACT CCC GCC CGT -3' Reverse: 5'- CGA CCT GCA GCA GCA CGT C -3'
<i>bfmK</i>	Forward: 5'- GCG GGT CGG TGT ACT GAA GG -3' Reverse: 5'- ATC GGC CAA GCG TCG TAG -3'

Statistical analysis

Data are expressed as means ± standard deviations (SD). Significant difference between treated and untreated samples was determined using Student's *t*-test. Statistical significance was considered at $p < 0.05$.

Results

Celastrol inhibited biofilm formation in *S. maltophilia* without affecting planktonic cell growth

To determine whether celastrol could affect biofilm formation in *S. maltophilia* strains (ATCC 13637 and GNU2233), the biofilm formation abilities were compared in the presence or absence of celastrol. Flat-bottom 96-well polystyrene microtiter plates were used. Phenotypic analyses were performed

using crystal violet based biomass staining assay. As shown in Figure 1, celastrol decreased biofilm formation in the two *S. maltophilia* strains in a dose-dependent manner, indicating that celastrol exhibited concentration-dependent anti-biofilm effects. The inhibitory effects of celastrol against biofilm formation in ATCC 13637 and GNU2233 were observed at concentrations of 5 and 20 µg/ml (biofilm reduced by 54.76 and 72.51%, respectively, compared to the control; Figure 1A and 1B). Planktonic cell growth of *S. maltophilia* was also evaluated to examine the anti-biofilm and anti-virulence activities of celastrol in the absence of its bactericidal activity. It was found that celastrol (at the concentrations tested) failed to exert any antimicrobial effects against the planktonic *S. maltophilia* cells (Figure 1C-1D). In this context, the cell growth of *S. maltophilia* in the presence of different concentrations of celastrol over different time periods indicated that celastrol exhibited a bacteriostatic effect. Furthermore, the antimicrobial activity of celastrol was evaluated by determining its minimum inhibitory concentration (MIC). The MICs of celastrol against *S. maltophilia* ATCC 13637 and GNU2233 were 20 and 80 µg/ml, respectively. It is noteworthy that celastrol (at the concentrations tested) exerted significant ($p < 0.05$)

inhibitory effects against biofilm formation in the *S. maltophilia* without reducing cell growth. These results indicated that the observed attenuation of biofilm formation exerted by celastrol was attributed to its anti-biofilm effects, not to its antimicrobial effects.

Dispersion of established *S. maltophilia* biofilms by celastrol

The biofilm dispersion activity of celastrol was investigated in *S. maltophilia* strains (ATCC 13637 and GNU2233) by measuring the biomass of biofilms. As shown in Figure 2, compared to the untreated controls (DMSO alone), celastrol treatment dose-dependently disassembled the established biofilms of *S. maltophilia* tested, indicating that celastrol exhibited concentration-dependent biofilm dispersion effects. In a more detailed study, celastrol exerted a significant ($p < 0.05$) dispersion activity against the established biofilms produced by *S. maltophilia* at all concentrations tested without inhibiting the growth of planktonic *S. maltophilia* cells. Therefore, celastrol inhibited and dispersed the biofilms produced by *S. maltophilia*. In addition, it significantly reduced the biomass of biofilms in a dose-dependent manner.

Effects of celastrol on the metabolism of established biofilms of *S. maltophilia* based on the XTT reduction assay

To further emphasize that celastrol could reduce the viability of biofilm cells, a colorimetric XTT reduction analysis was performed after treatment of the established biofilms with celastrol. Results of the XTT reduction assay revealed that the metabolic activity of *S. maltophilia* strains (ATCC 13637 and GNU2233) decreased with increasing the concentration of celastrol ($p < 0.05$, Figure 3). After 24-hour exposure of *S. maltophilia* biofilms to celastrol at concentrations of 80 and 100 µg/ml, the specific absorbance values were significantly ($p < 0.05$) reduced, compared to that of the DMSO-treated control group (Figure 3). In particular, celastrol at 80 µg/ml significantly decreased the metabolic activity of *S. maltophilia* ATCC 13637 biofilm cells by more than 80%, compared to the DMSO-treated control (Figure 3A). At a concentration of 100 µg/ml, celastrol diminished the viability of GNU2233 biofilm cells by more than 90% (Figure 3B).

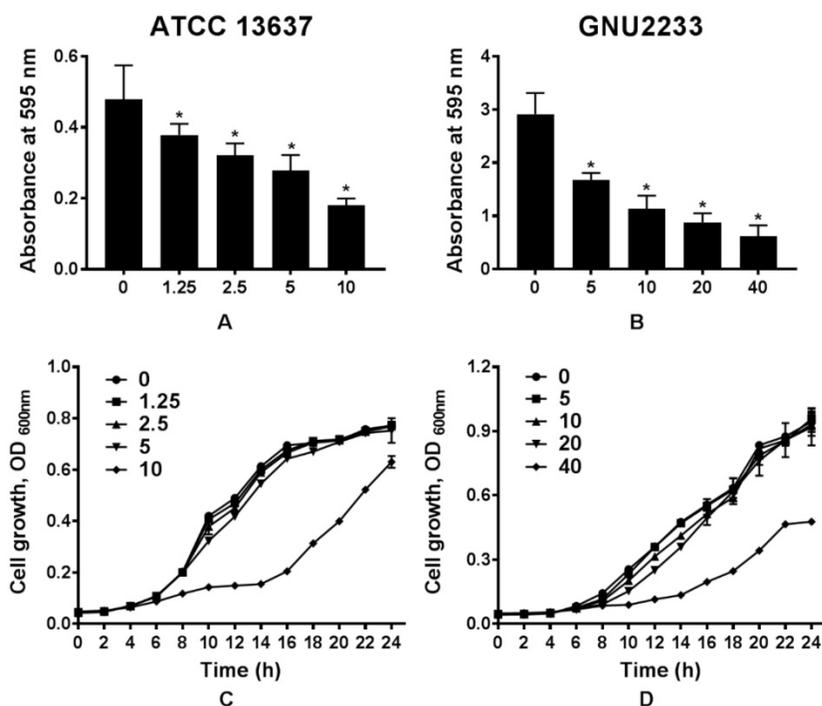


Figure 1. Effect of celastrol on biofilm formation of *S. maltophilia*. Biofilms produced by *S. maltophilia* strains ((A) ATCC 13637 and (B) GNU2233) were quantified by measuring the absorbance values at OD595nm in the presence or absence of celastrol at increasing doses. Planktonic cell growth of *S. maltophilia* strains ((C) ATCC 13637 and (D) GNU2233) in the presence of celastrol was determined by measuring absorbance at wavelength of 600 nm. Mean values of triplicate independent studies are shown. Error bars represent standard deviations (SDs). Asterisks (*) indicate statistically significant change ($p < 0.05$) compared to that of DMSO treated control.

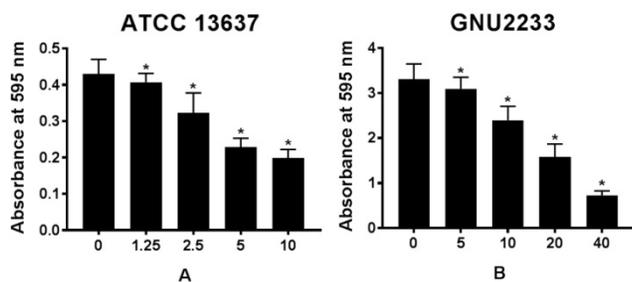


Figure 2. Effect of celastrol on established biofilms formed by *S. maltophilia* strains (A) ATCC 13637 and (B) GNU2233). To determine the ability of celastrol to disrupt mature biofilms, *S. maltophilia* strains were cultured at 37 °C for 24 h to form established biofilms in microtitre plates. Celastrol was then added at different concentrations. These cultures were incubated further for 24 h. Total biofilm formation of *S. maltophilia* strains were examined by measuring absorbance at OD595nm. Mean values of triplicate independent studies are shown. Error bars represent standard deviations (SDs). Asterisks (*) indicate statistically significant change ($p < 0.05$) compared to that of DMSO treated control.

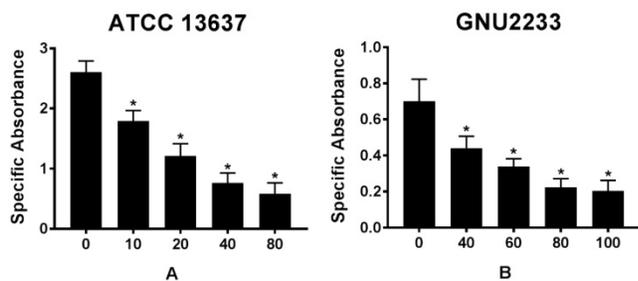


Figure 3. Effect of celastrol on metabolic activities of established biofilms formed by *S. maltophilia* strains (A) ATCC 13637 and (B) GNU2233). The viability of cells within the biofilms treated with or without celastrol was evaluated by XTT viability assay. Specific absorbance indicates $A_{490nm}(\text{Test}) - A_{490nm}(\text{Blank}) - A_{655nm}(\text{Test})$. Mean values of triplicate independent studies are shown. Error bars represent standard deviations (SDs). Asterisks (*) indicate statistically significant change ($p < 0.05$) compared to that of DMSO treated control.

Effects of celastrol on biofilm structure

To further confirm our findings regarding the effects of celastrol on the established biofilms formed by *S. maltophilia* ATCC 13637, biofilm matrix components were individually stained with FITC, concanavalin A, and DAPI, followed by characterization using confocal laser scanning microscopy (CLSM). As shown in the CLSM images, biofilms treated with celastrol at concentrations of 40 and 80 µg/ml exhibited a significantly different structure, compared to that of the DMSO-treated control (Figure 4), which is consistent with the results of the 96-well biofilm assays (Figure 2 and 3). Micrographs of DMSO-treated control (Figure 4A) showed a spatial biomass distribution of the formed biofilms and a relatively thick coating of the biofilms with well-organized architecture characterized by large clumps over a glass surface. However, after treatment with celastrol (Figure 4B and 4C), the established biofilms were disrupted and scattered as microcolonies, wherein the biofilm cells remained-in-

the form of elongated chains, indicating that cells within the formed biofilms were probably challenged, killed, and detached from the established biofilms. It has been reported that phenotypic heterogeneity can occur in response to stressors, including antibiotic treatment. This is one of the mechanisms used by the bacteria to survive the changes in the environment [30]. In addition, the other study showed that phenotypic heterogeneity was observed when *S. maltophilia* K279a was challenged with ampicillin, resulting in the formation of long cell chains [31]. The results of this study are consistent with our findings in *S. maltophilia* ATCC 13637 after treatment with celastrol. Because microbial biofilms were formed at the bottom and sides of the 96-well plates, CLSM was used to visualize the biofilms formed on the glass bottom plates. Microscopic examination showed that celastrol at 40 and 80 µg/ml markedly eradicated the biofilms formed on the glass bottom surface. It also penetrated the biofilm, eventually killing the bacteria.

Celastrol reduced the swimming motility of *S. maltophilia*

The effects of celastrol on the swimming motility of *S. maltophilia* strains (ATCC 13637 and GNU2233) were examined in a phenotypic study using semisolid agar. As shown in Figure 5, after 24-hour incubation, *S. maltophilia* showed a significant ($p < 0.05$) decrease in the diameter of the swimming zones in the agar plate containing celastrol. Celastrol decreased the diameter in a dose-dependent manner. Since the concentrations of celastrol tested were sub-inhibitory concentrations, the reduction of swimming motility could not be attributed to growth disturbance. In particular, the swimming motility zone of ATCC 13637 strain treated with celastrol at 5 µg/ml was 9 mm in diameter, which was smaller than that of the DMSO-treated control by 47.06%. In addition, celastrol at 20 µg/ml reduced the swimming motility of GNU2233 strain by more than 31.65%. Therefore, celastrol at sub-inhibitory concentrations reduced the swimming motility of both *S. maltophilia* strains.

Celastrol inhibited protease secretion by *S. maltophilia*

To investigate the effects of celastrol on the secretion of extracellular protease, another virulence-associated factor of *S. maltophilia*, a phenotypic assay was performed using a casein agar plate. After 48-hour incubation in casein agar plate, the hydrolysis zone formed around the inoculated supernatant was measured. As shown in Figure 6, celastrol at sub-inhibitory concentrations dose-dependently inhibited ($p < 0.05$) the proteolytic activity of *S. maltophilia* strains (ATCC 13637 and

GNU2233). Notably, celastrol at 5 $\mu\text{g}/\text{ml}$ significantly ($p < 0.05$) reduced the diameters of the halo zones of the ATCC 13637 strain by more than 49.1%, compared to the DMSO-treated control. In addition, *S. maltophilia* GNU2233 treated with celastrol at 20 $\mu\text{g}/\text{ml}$ showed a decrease in the proteolytic activity by approximately 13.62%. These results showed that celastrol at sub-inhibitory concentrations inhibited protease secretion by *S. maltophilia* strains.

Celastrol induced transcriptional changes in virulence- and biofilm-related genes of *S. maltophilia*

To investigate the molecular mechanisms of the anti-biofilm and anti-virulence activities of celastrol, qRT-PCR was performed to measure the expression of the genes (Table 1) related to biofilm formation and virulence in both celastrol-treated and DMSO-treated *S. maltophilia* cells (ATCC 13637 and GNU2233). Celastrol affected the expression of many genes, compared to the internal housekeeping control gene (Figure 7). In consistence with the results of inhibition of biofilm formation (Figure 1) and attenuation of

virulence-related characteristics (Figure 5 and 6), celastrol significantly reduced the gene expression of *smeYZ*, encoding SmeYZ efflux pump (involved in swimming, protease secretion, and biofilm formation), by more than 30- and 3-fold in the ATCC 13637 and GNU2233 strains, respectively (Figure 7A and 7B, respectively; $p < 0.05$). In addition, celastrol significantly decreased the gene expression of *fsnR*, encoding the orphan response regulator FsnR (involved in motility and biofilm formation), by more than 10- and 3-fold in the ATCC 13637 and GNU2233 strains, respectively (Figure 7A and 7B, respectively; $p < 0.05$). Furthermore, the expression of two HK genes, encoding the BfmAK system involved in biofilm formation and development, was significantly down-regulated by celastrol by more than 3-fold in both *S. maltophilia* strains (Figure 7Aa and 7B; $p < 0.05$). Overall, the repression of these genes by celastrol confirmed the observed inhibitory effects of celastrol against the virulence factors and biofilm formation in *S. maltophilia*.

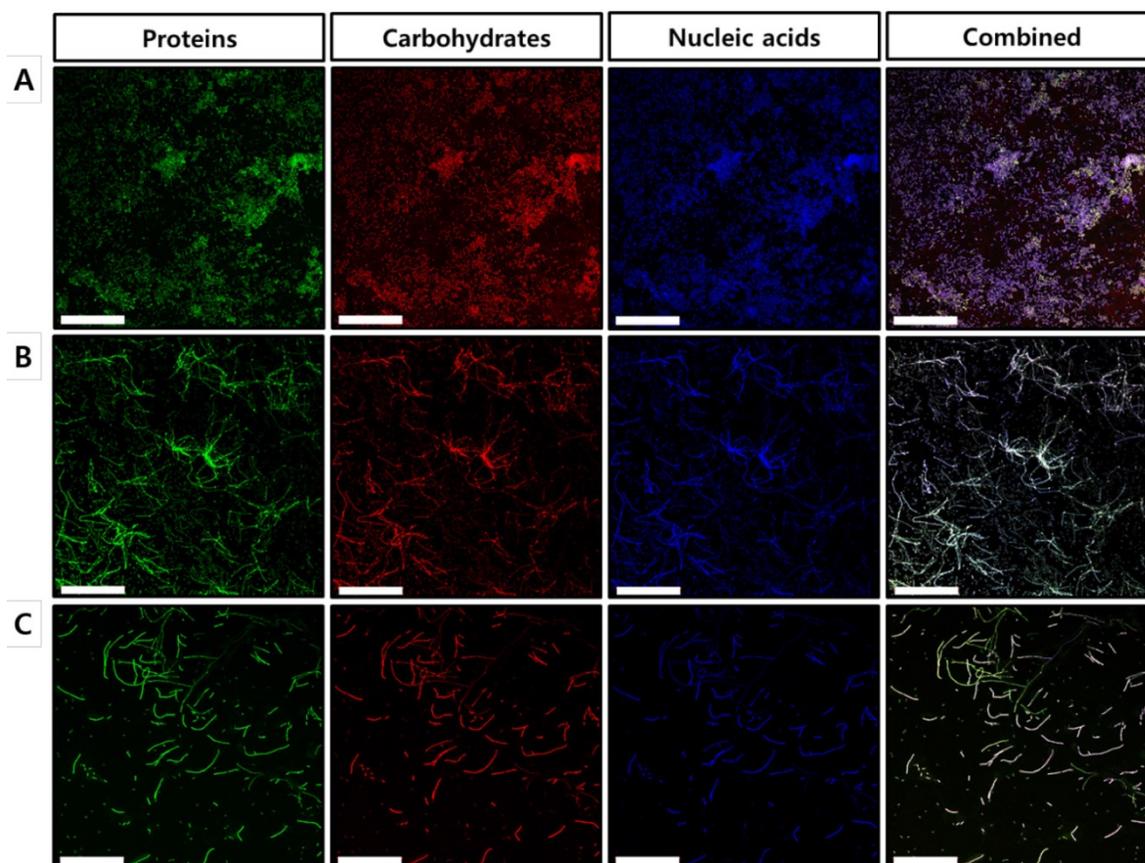


Figure 4. Microscopic evaluation of the effect of celastrol on established biofilms formed on glass surface using a confocal laser scanning microscope. CLSM micrographs of *S. maltophilia* ATCC 13637 biofilms treated with (A) DMSO 1 % (v/v) (control) and celastrol at concentration of (B) 40 $\mu\text{g}/\text{mL}$ or (B) 80 $\mu\text{g}/\text{mL}$. Green (FITC) and red (Concanavalin A) colors indicate proteins and carbohydrates of EPS, respectively. Blue fluorescent staining (DAPI) represents extracellular DNA. CLSM images of panel (A) of DMSO-treated control *S. maltophilia* biofilms have spatial biomass distribution of mature biofilms and thick coating of biofilm with compact architecture characterized by large clumps. On the other hand, in panels (B) and (C) CLSM images of *S. maltophilia* biofilm treated with celastrol at 40 and 80 $\mu\text{g}/\text{mL}$, the established biofilms are almost disrupted and biofilm cells are remained as long cell chains. Magnification: 400x for each dose. Scale bar: 50 μm .

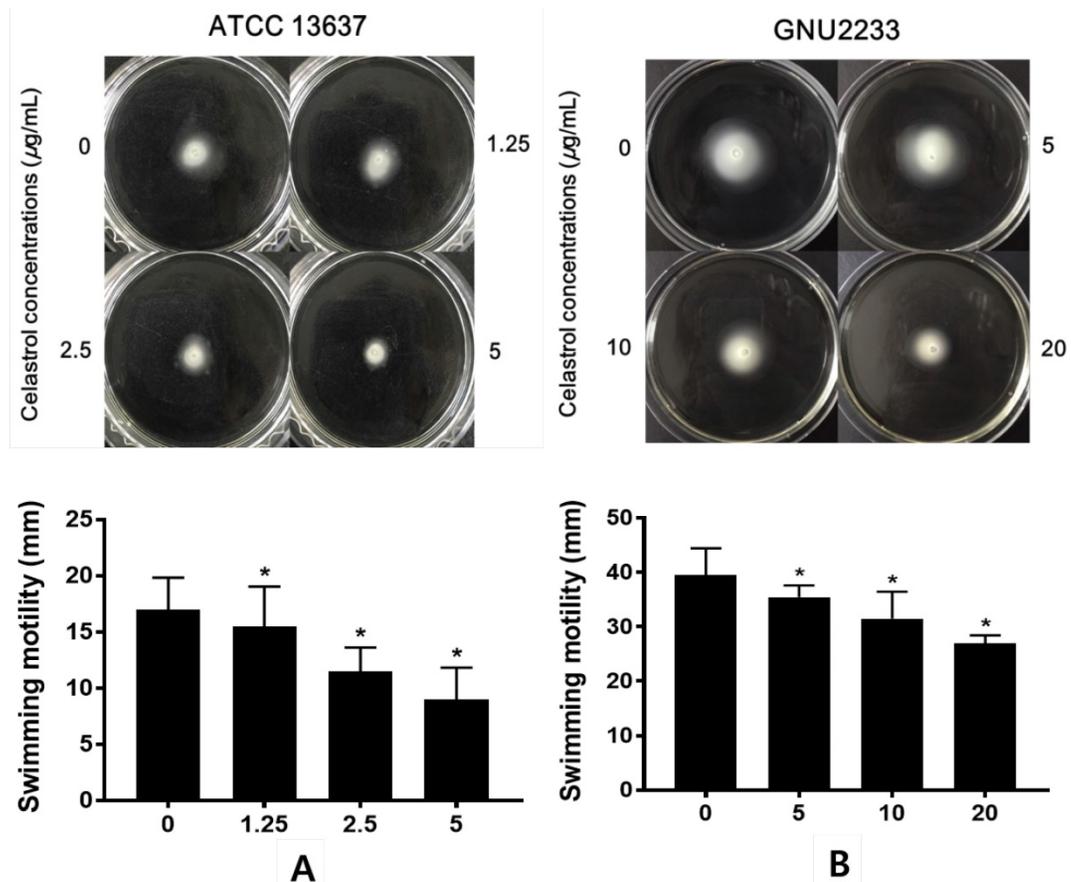


Figure 5. Effect of celastrol on motility of *S. maltophilia*. Motility assays were performed using 0.15% agar plate containing 1% tryptone and 0.5% NaCl. (A) ATCC 13637 and (B) GNU2233 strains were inoculated in the center of motility agar surface including DMSO 1% (v/v) or celastrol at sub-inhibitory concentrations. The inoculated plates were incubated at 37 °C and the diameter (mm) of circular swimming zone was measured at 24 h. Mean values of triplicate independent studies are shown. Error bars represent standard deviations (SDs). Asterisks (*) indicate statistically significant change ($p < 0.05$) compared to that of DMSO treated control.

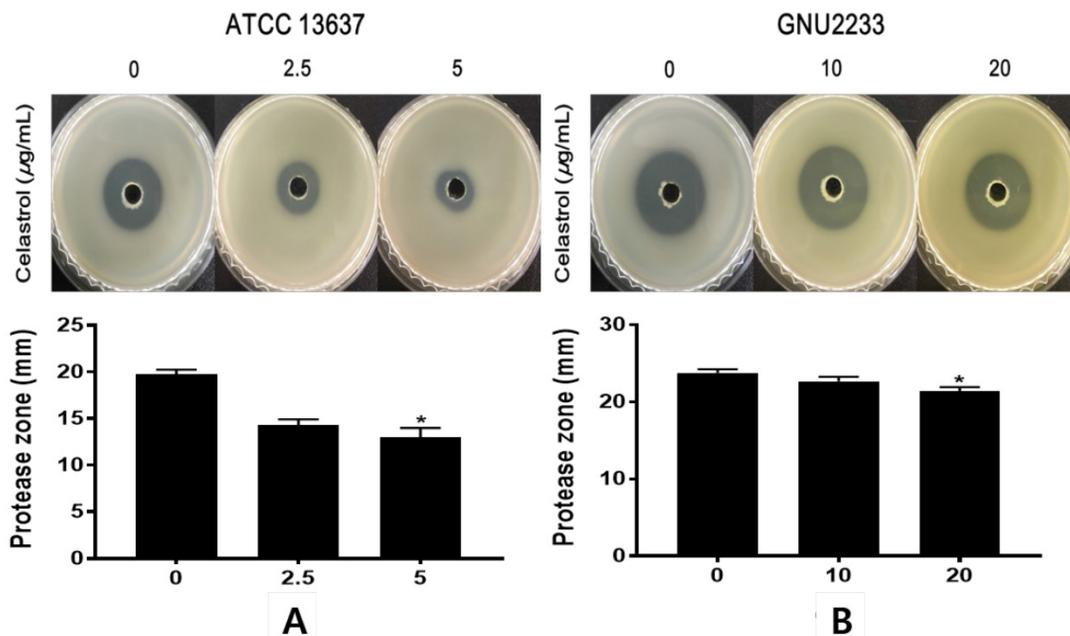


Figure 6. Effect of celastrol on protease production of *S. maltophilia*. Proteolytic activity was determined using casein agar. (A) ATCC 13637 and (B) GNU2233 cells were incubated with or without sub-inhibitory concentrations of celastrol at 37 °C for 24 h. Secreted protease assays were performed using culture supernatants. Diameters of transparent zones surrounding the holes were measured at 48 h. Mean values of triplicate independent studies are shown. Error bars represent standard deviations (SDs). Asterisks (*) indicate statistically significant change ($p < 0.05$) compared to DMSO treated controls.

Discussion

The ever increasing emergence of multidrug resistant opportunistic pathogens, such as *S. maltophilia*, in clinical settings has become a worldwide public health problem owing to the lack of effective treatment measures, its intrinsic multidrug resistance, antibiotic-modifying enzymes, and antibiotic-resistant biofilm formation. Furthermore, superbugs such as *S. maltophilia* can cause chronic infections through formation of biofilms, which are difficult to encounter with conventional antibiotics [3]. Thus, discovery and development of alternative anti-pathogenic agents is urgently needed to reduce the emergence of antibiotic resistance and overcome life-threatening infections caused by antibiotic-resistant organisms. This alternative approach, unlike the conventional antibiotics that aim to inhibit cell growth, can combat the pathogenesis of biofilm-related infections caused by multidrug resistant pathogens without increasing the risk of drug resistance emergence [32]. The present study provided phenotypic and genotypic evidence that celastrol could be a potential anti-pathogenic agent for treatment of *S. maltophilia* infections since it showed significant anti-biofilm and anti-virulence activities against *S. maltophilia* without affecting their planktonic cell growth.

Recent pharmacological and clinical studies have shown that friedelane-type triterpenoids exert various pharmacological effects, including anti-oxidant, anti-tumor, anti-inflammatory, anti-HIV, and anti-bacterial bioactivities [33]. Herein, at sub-inhibitory concentrations, celastrol was not lethal to *S.*

maltophilia. It dose-dependently decreased biofilm formation, motility, and protease secretion by *S. maltophilia*. Additionally, celastrol disrupted the previously established biofilms and interfered with the expression of biofilm- and virulence-associated genes.

The mechanisms underlying biofilm formation in *S. maltophilia* involve a complex network modulated by environmental factors, including temperature, phosphate, pH, antibiotics, metal ions, and other factors [3]. Moreover, *S. maltophilia* genome contains diverse array of multidrug efflux pumps and many other molecular machineries that can increase the resistance of *S. maltophilia* to various antibiotics [6]. In this regard, *S. maltophilia* infections have become a critical threat to human health.

To gain further insights into whether celastrol could inhibit the molecular mechanisms involved in biofilm formation and repress the expression of virulence-associated genes in *S. maltophilia*, the transcription levels of various genes were examined using qRT-PCR analysis. As expected, biofilm and virulence regulators (*SmeYZ*, *FsnR*, and *BfmAK* system) were significantly down-regulated by celastrol (Figure 7), resulting in reduced biofilm formation (Figure 1) and attenuated virulence-related characteristics (Figure 5 and 6), which validated the phenotypic effects of celastrol.

It has been shown that the RND-type efflux pump, *SmeYZ* contributes to intrinsic and acquired multidrug resistance to aminoglycosides and trimethoprim-sulfamethoxazole owing to its antimicrobial extrusion functions [34]. In addition, it is involved in biofilm formation, flagella formation, oxidative stress susceptibility, swimming motility, and protease secretion [8], which supported our findings that suppression of *smeYZ* gene expression (Figure 7) correlated with the attenuation of biofilm formation (Figure 1), swimming motility (Figure 5), and protease secretion (Figure 6).

A previous study has reported that *fsnR* gene encodes the orphan response regulator, *FsnR*. Genetic and biochemical evidence suggested that *FsnR* could trigger the transcription of at least two flagella-related operons and mediate cell motility and biofilm formation. Suppression of *fsnR* gene expression is a crucial strategy to inhibit *S. maltophilia* cell motility and biofilm formation [19]. Our results of the transcriptional analysis showed

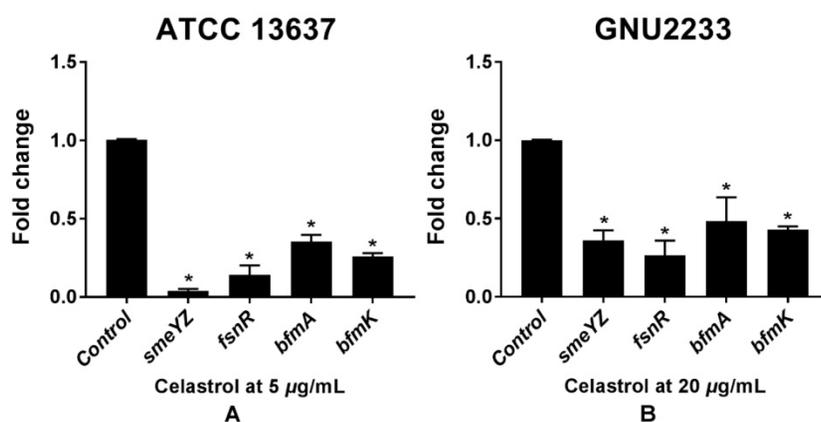


Figure 7. Transcriptional profiles of *S. maltophilia* cells treated with or without celastrol. ATCC 13637 and GNU 2233 were cultivated in CA-MHB medium at 37 °C for 6 h with shaking (250 rpm). Thereafter, they were further incubated at 37 °C for 4 h with or without celastrol at (A) 5 µg/mL (ATCC 13637) or (B) 20 µg/mL (GNU2233) without shaking. Transcriptional profiles were examined by qRT-PCR analysis. Relative gene expressions indicate transcriptional levels after treatment with celastrol versus DMSO treated control (value of 1.0). The tmRNA was used to normalize the expression levels of genes tested. All measurements were conducted with three individual cultures. Additionally, three replications were used for each qRT-PCR reaction. Mean values of triplicate independent studies are shown. Error bars represent standard deviations (SDs). Asterisks (*) indicate statistically significant change ($p < 0.05$) compared to that of DMSO treated control.

that celastrol inhibited the expression of *fsnR* gene (Figure 7). These findings suggested that down-regulation of *fsnR* gene expression could interfere with the biofilm formation ability of *S. maltophilia*, thus eradicating *S. maltophilia* infections, particularly those biofilm-related infections.

A recent study has suggested that two HK genes encode a two-component TCS, the BfmAK system. This system has been found to play multiple roles in the formation of *S. maltophilia* biofilms [20]. BfmA has been found to be a transcription factor that stimulates the transcription of *bfmA-bfmK* operon and *smlt0800* (*acoT*, a gene encoding acyl coenzyme A thioesterase) by directly binding to their promoter regions; in addition, it has been linked to biofilm development [20]. Based on our results, inhibition of *bfmA* and *bfmK* gene expression by celastrol possibly contributed to the reduction of biofilm formation in *S. maltophilia* (Figure 1 and 7). These findings suggested that celastrol could inhibit the expression of several important biofilm- and virulence- regulating genes in *S. maltophilia*.

When mature biofilms are completely formed by dreadful biofilm-forming pathogens, conventional antibiotics usually fail to eradicate the infection because the multilayer architecture of the biofilm acts as a diffusion barrier. This can provide protection to biofilm-grown cells against antimicrobial agents or antibiotics. Therefore, high biofilm disrupting concentrations are required [35], and strategies focusing on destroying the formed biofilms are of particular importance. The effects of celastrol on the established biofilms of *S. maltophilia* strains were further tested using microtiter plate assay and CLSM. Results revealed that celastrol not only induced disruption of the complex architecture of the biofilms but also killed many sessile cells within the biofilms by loosening the microcolonies. It is plausible to infer that this is linked to increased antibiotic penetration promoted by celastrol into the established biofilms of *S. maltophilia*. It is noteworthy that this does make clinical sense; otherwise, biofilm-related clinical infections will proceed without prevention.

To counter the dreadful pathogenic bacteria, antibiotics, which can induce resistance emergence, have been used for a long time. Therefore, novel strategies are urgently needed to control antibiotic-resistant *S. maltophilia*. To decrease the bacterial virulence and overcome biofilm-associated resistance, prevention of biofilm formation, eradication of pre-existing biofilms, and halting of the ongoing virulence factor production could be used as alternative control strategies. Results of this study showed that celastrol, a pentacyclic triterpenoid, possessed anti-biofilm and anti-virulence activities. It

also inhibited the expression of genes related to biofilm formation and virulence of *S. maltophilia*.

Overall, celastrol might be a potential anti-pathogenic agent by targeting biofilm formation and virulence factors without inhibiting bacterial growth. It could be administered in combination with antibiotics or other bactericides to induce synergistic effects and achieve better control of persistent *S. maltophilia* infections. In addition, the anti-biofilm and anti-virulence activities of celastrol demonstrated the value of plant-derived compounds as promising and potent bioactive agents.

Abbreviations

ATCC: American type culture collection; CA-MHB: cation-adjusted Mueller Hinton broth; CLSM: confocal laser scanning microscopy; ConA: Concanavalin A; DAPI: 4',6-diamidino-2-phenylindole dihydrochloride; DMSO: dimethyl sulfoxide; FITC: fluorescein isothiocyanate; MIC: minimum inhibitory concentration; PBS: phosphate buffered saline; qRT-PCR: quantitative reverse transcription-polymerase chain reaction; RND: resistance-nodulation-division.

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Competing Interests

The authors have declared that no competing interest exists.

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