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Research article

Quorum sensing inhibition properties of lichen forming fungi extracts from *Cetrelia* species against *Pseudomonas aeruginosa*

Baris Gokalsin[1], Didem Berber[2], Jae-Seoun Hur[3], Nuzhet Cenk Sesal[*2]

[1] *Marmara University, Institute of Pure and Applied Sciences, Department of Biology, Istanbul, Turkey*
[2] *Marmara University, Faculty of Arts and Sciences, Department of Biology, Istanbul, Turkey*
[3] *Sunchon National University, Korean Lichen Research Institute, Suncheon, Republic of Korea*

Abstract

Pseudomonas aeruginosa is known to be responsible for many antibiotic resistant infections in hospitals. It can regulate its virulence gene expressions through its QS systems. It is now known that QS inhibition can make bacteria less violent and more vulnerable. Many natural sources such as bacteria, fungi and plants are known to produce QS inhibitor (QSI) metabolites. This study aims to investigate the QSI bioactivity of culture extracts obtained from lichen-forming fungi (LFF) of five different *Cetrelia* species against *P. aeruginosa.* Extracts were applied to monitor strains and all samples have shown varying amounts of QS inhibition activity. Our study demonstrates that LFF cultures can be utilized to produce QSI compounds instead of collecting slow growing natural lichen thalli. We believe that this research will cast a new light on identification and isolation of active compounds from the extracts and assessment of their compatibility for future drug research.

Keywords: *Quorum sensing; Pseudomonas aeruginosa; secondary metabolites; lichen forming fungi*

1. Introduction

Pseudomonas aeruginosa is a Gram negative, opportunistic nosocomial pathogen bacterium associated with multidrug resistant infections in immune compromised patients (Jones et al., 2009; Lambert et al., 2011; Pachori et al., 2019). *P. aeruginosa* is stated as the leading cause for high mortality rates in cystic fibrosis patients (Ciofu et al., 2015).

It can also survive in various environmental conditions due to its biofilm form, which provides a stable and secure environment to resist extreme conditions such as exposure to UV, antibiotics, pH changes or antimicrobial agents (Brackman and Coenye, 2015). Therefore, it is imperative to prevent the ability to develop biofilms, especially in antibiotic resistant strains in hospitals. *P. aeruginosa* employs cell-to-cell signaling mechanism called *quorum* sensing (QS) to regulate its physiological behaviors such as biofilm production, virulence and motility (Kostylev et al., 2019).

All bacteria communicate with each other via QS, utilizing small diffusible signaling molecules called autoinducers (AIs), leading to sporulation, conjugation, biofilm formation, bacteriocin production, bioluminescence, swarming etc. (Li et al., 2016). QS is mediated by AI molecules called N-acyl-homoserine lactones (AHLs) in Gram negative bacteria and by autoinducer peptides (AIP) in Gram positive bacteria (Jakobsen et al., 2013). Autoinducer-2 (AI-2) signal molecules have been detected in both Gram positive and negative bacteria. AHLs diffuse through the cell membrane and bind to regulatory proteins within the cell in Gram negative bacteria including *P. aeruginosa.*

The AHL-mediated QS system has been well characterized in *P. aeruginosa*, which has two main QS systems called LasIR

* Corresponding author.
E-mail address: csesal@marmara.edu.tr (N. C. Sesal).

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and RhlIR. LasI produces *N*-(3-oxododecanoyl)-L-homoserine, lactone (3-oxo-C12-HSL) and RhlI produces *N*-butanoyl homoserine lactone (C4-HSL). These signal molecules accumulate in the surrounding milieu and activate transcriptional regulators LasR and RhlR. Pseudomonas quinolone signal (PQS) system is believed to act as a mediatory between LasIR and RhlIR QS systems (Diggle et al., 2003; McGrath et al., 2004).

The novel approach by employing quorum sensing inhibitors (QSIs) provides a fighting chance against bacteria before they develop antibiotic resistance (Hentzer et al., 2003). It is shown that inhibiting QS can leave bacteria vulnerable (Zeng et al., 2008). It is expected that natural metabolites can suppress QS systems of bacteria and thus they are a noteworthy source for drug materials (Paczkowski et al., 2017). In a recent study, Ahmed et al., (2019) have shown that *trans*-cinnamaldehyde and salicylic acid inhibit *P. aeruginosa* QS and thus reduce its virulence.

Lichens are complex organisms consisting of algae or cyanobacteria (photobiont) living together with fungi (mycobiont) in a symbiotic relationship. They are found in a wide range of habitats and synthesize chemically diverse organic compounds derived from primary or secondary metabolism (Molnar and Farkas, 2010). Secondary metabolites have importance in ethnobotany for their medicinal properties (Lal and Upreti, 1995; Malhotra et al., 2008). More than 1050 secondary metabolites are known, and their bioactivities such as antitumor, antibacterial, antifungal, antiviral, anti-inflammatory and antioxidant activities have been demonstrated (Molnar and Farkas, 2010; Shukla et al., 2010). It has been explained that lichens are promising candidates for discovering novel QSIs (Gokalsin and Sesal, 2016).

The lichen genus *Cetrelia* W.L. Culb. & C.F. Culb. belongs to lichenized fungi *Parmeliaceae* family. Like most secondary metabolites in lichens, the production of these compounds are regulated by polyketide synthase (PKS) of mycobiont counterpart. It is assumed that culturing the lichen forming fungi (LFF) has better potential for producing secondary metabolites in laboratory conditions than harvesting lichen thalli in nature. Therefore, for this study LFF cultures of five *Cetrelia* species were obtained from Korean Lichen & Allied Bioresource Center (KoLABIC), Sunchon National University, Korea, to investigate their QS inhibition potentials against *P. aeruginosa.* For this purpose, extracts were obtained from LFF cultures of *Cetrelia* species, and fluorescent monitor strains of *P. aeruginosa* were utilized for inhibition analyses.

2. Materials and Methods

2.1. Bacteria strains

P. aeruginosa lasB-gfp and *rhlA-gfp* biomonitor strains were employed to quantify QS inhibition (Hentzer et al., 2002; Yang et al., 2009). They contain promoters for two main QS systems that produce green fluorescent protein (GFP). The bacteria were grown in LB, and the QS inhibition assays were conducted with M9 minimal media (with 2.5 mg/l thiamine, 0.5% glucose, 0.5% casamino acids).

2.2. LFF isolation and cultures

Spore-discharge method (Yamamoto, 2002) was used to isolate the LFF from five *Cetrelia* species: *Cetrelia japonica, C. olivetorum, C. braunsiana, C. chicitae* and *C. delavayana.* All lichens were collected and the fungi were isolated by KOLABIC. Culture information is shown in Table 1.

Table 1
Information on lichen collections and their LFF cultures with the applied extract concentrations.

Lichen name	Collection No.	Country	KOLABIC No. (Lichen)	Extract concentrations (µg/ml)
Cetrelia japonica	30397	Korea	330	65
C. olivetorum	CH050076	China	3725	33.3
C. braunsiana	40425	Korea	1203	24.7
C. chicitae	TW090067	Taiwan	9612	16.7
C. delavayana	06-26823	China	8128	16

2.3. Preparation of LFF extracts

Approximately 1 g fresh weight mycelia were inoculated to malt-yeast agar plates. Grown LFF were then gently crushed and transferred into malt-yeast medium. All LFF samples were cultured at 18°C for 3 months (150 rpm shaking). Culture media were filtered and the filtrates were extracted by ethyl acetate 3 times. Solvents were removed using a rotary evaporator, and the powder extracts were weighed.

2.4. Pseudomonas aeruginosa QSI screens

QS inhibition assays were performed according to Bjarnsholt et al., (2010), with slight modifications. In short, M9 media (with 2.5 mg/l thiamine, 0.5% glucose, 0.5% casamino acids) were added to clear bottom black 96-well plates and two-fold serial dilutions of the extracts were prepared. Strains grown overnight were then added to obtain a final OD of 0.1 at 450 nm. Applied concentrations of the LFF extracts are shown in Table 1. Absorbance (450 and 600 nm) and fluorescence intensities (Ex: 485 nm, Em: 535 nm) were measured using a microplate reader (Cytation 3, Biotek), every 15 minutes, with constant shaking (180 rpm) at 36 °C for 16 hours.

2.5. HPLC Analysis

Ethyl-acetate extracts were analyzed by HPLC (LC-10AT, Shimadzu, Japan) under following conditions: Acetonitrile (ACN) in 0.1% trifluoroacetic acid (TFA) gradient; solvent, Methanol:Chloroform (1:1, v:v); 1 ml/min flow rate; photodiode array detector (range 180-700 nm); detecting wavelength: 254 nm.

3. Results

3.1. QSI activity

LFF extracts obtained from five different *Cetrelia* species were applied to the monitor strains with the final concentrations as shown in Table 1. According to the results, all extracts have shown QSI properties on the monitor strain *lasB-gfp* and *rhlA-gfp* (Fig. 1-2).

The results are means of 3 individual experiments. Compared to untreated groups, the highest QS inhibition was observed in the LFF extract of *C. braunsiana* (approximately 63.8% for *las* and 52.6% for *rhl* system). Other samples also show similar inhibition percentages at different concentrations.

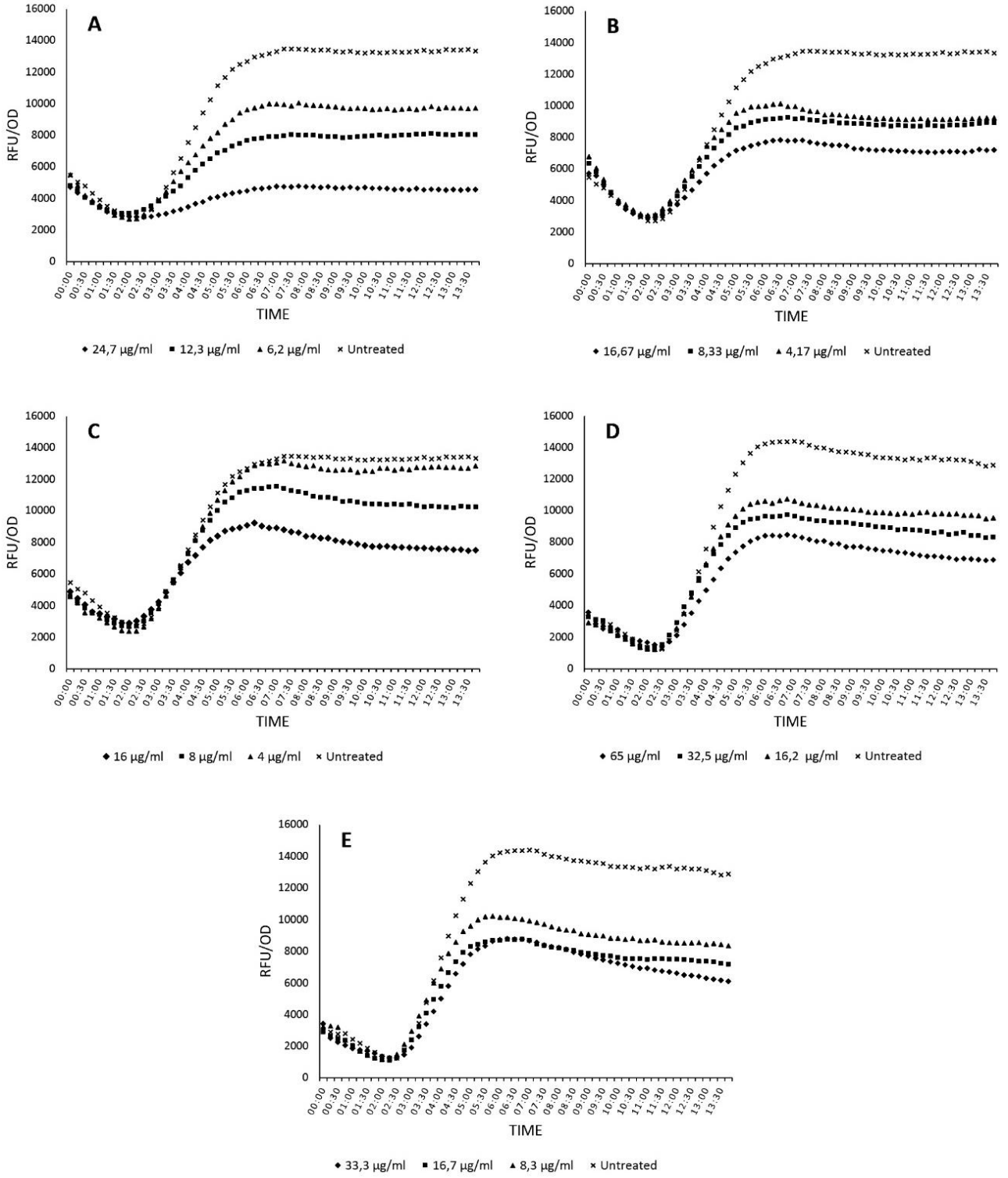

Fig. 1. Dose response curves of *lasB-gfp* monitor strain treated with LFF extracts obtained from different *Cetrelia* species. Data are shown as Relative Fluorescence Unit over Optical Density of 450nm. (A) *C. braunsiana*, (B) *C. chicitae*, (C) *C. delavayana*, (D) *C. japonica* and (E) *C. olivetorum*.

Results are presented as relative fluorescence unit (RFU) over absorbance to take minor growth inhibition rates into account. Descriptive statistics were used in this study.

2.3. HPLC Analysis

According to 16th minute retention time of HPLC, a common absorbance peak was detected in all extracts except *C. chicitae*. Results are shown in Fig. 3.

4. Discussion

P. aeruginosa continues to be a major cause of nosocomial infections in hospitals. It can detect its surrounding population using a signal system called QS and collectively res-pond to this information by regulating its virulence gene expressions (De Kievit, 2009). Employing QSIs proved that biofilm formation and virulence can be inhibited and provide a fighting chance against bacteria (Hancock and Speert, 2000).

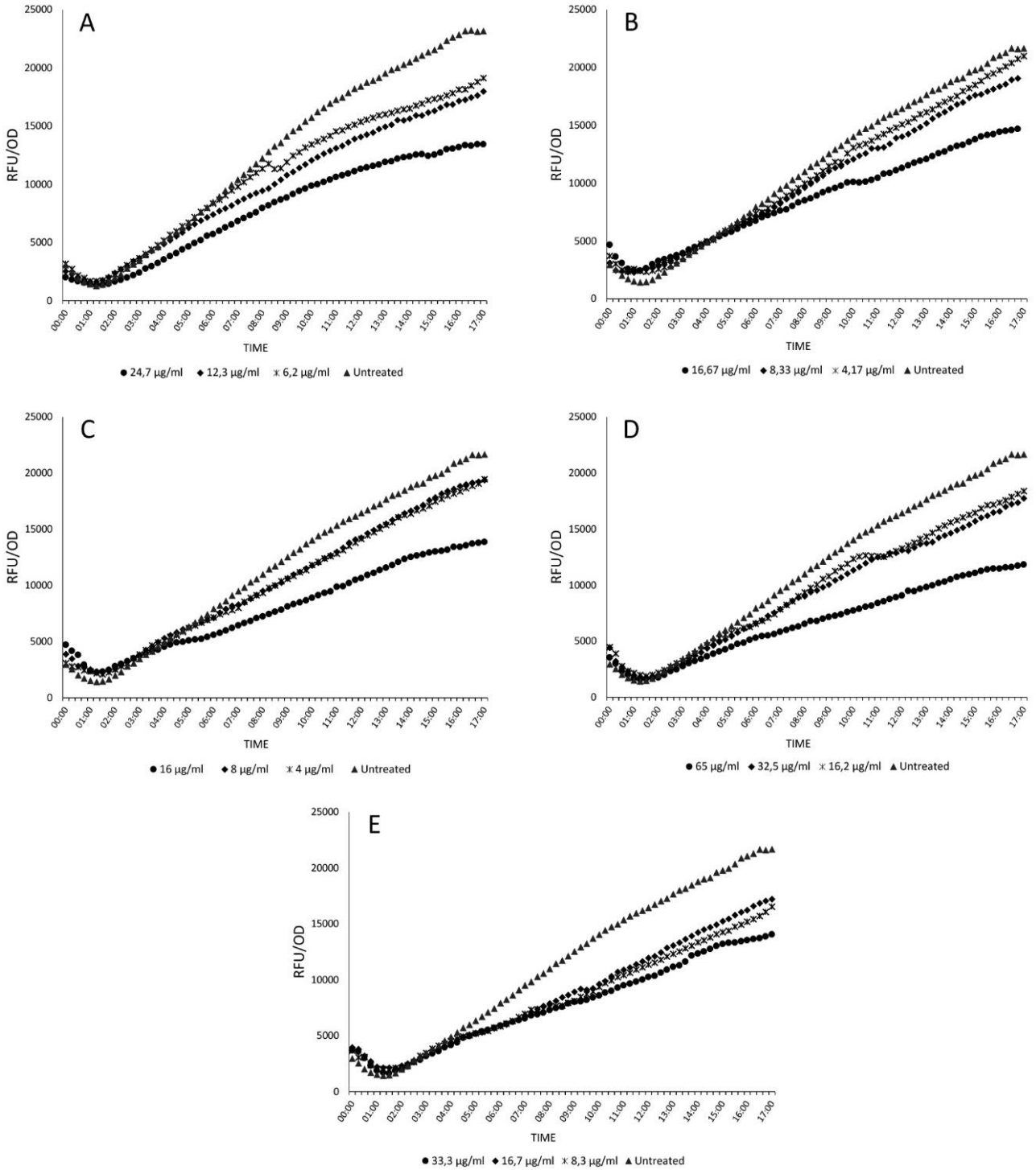

Fig. 2. Dose response curves of *rhlA-gfp* monitor strain treated with LFF extracts obtained from different *Cetrelia* species. Data are shown as Relative Fluorescence Unit over Optical Density of 450nm. (A) *C. braunsiana,* (B) *C. chicitae,* (C) *C. delavayana,* (D) *C. japonica* and (E) *C. olivetorum.*

Many effective QSIs have been discovered to this day (Azimi et al., 2012; Truchado et al., 2012; Al-Ani et al., 2015; Savo et al., 2015). New potential QSIs from lichen biosources would expectedly aid this possible alternative treatment method.

This study aims to investigate the QSI bioactivity of extracts obtained from LFF cultures of five *Cetrelia* species against *P. aeruginosa.* For this purpose, concentrations of ethyl-acetate extracts were applied to GFP producing monitor strains *lasB-gfp* and *rhlA-gfp* to observe QS inhibition. According to the results, all LFF isolates in this study have shown QSI properties with varying amounts. This means that every *Cetrelia* species in this study contains secondary metabolites that are biologically active against *P. aeruginosa* QS; although it is debatable if they're the same or similar compounds. According to HPLC analyses, a common compound exists in the LFF extracts of *C. japonica, C. olivetorum, C. braunsiana,* and *C. delavayana.* This unidentified compound might be the reason for QSI activity in these extracts. However, the amount of this compound in each species would most certainly change resulting in varying QS inhibition properties.

In a study, Savale et al., (2016) have shown that methanol extracts of C. *olvietorum* and its culture has antimicrobial pro-

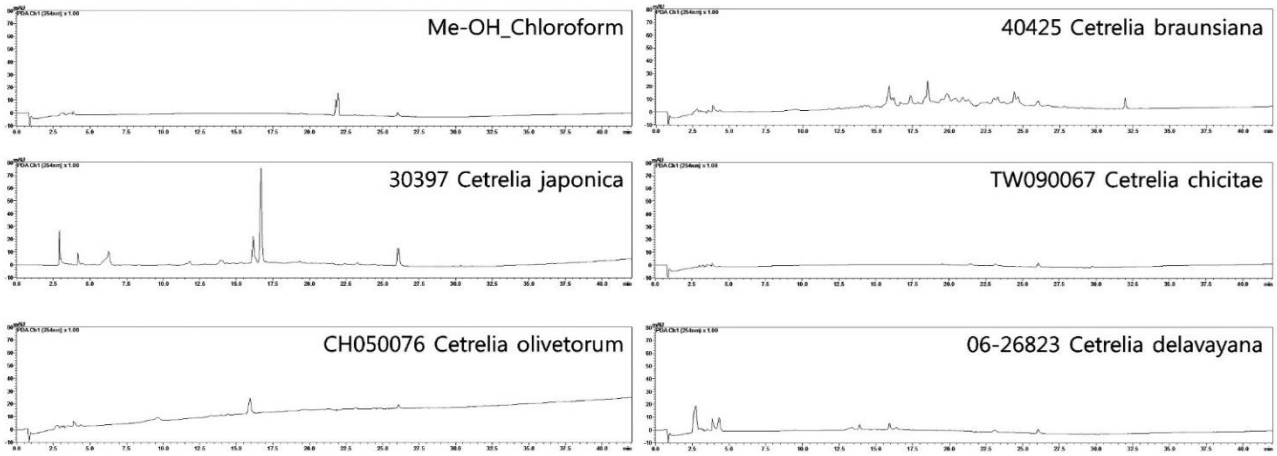

Fig. 3. HPLC chromatograms of ethyl-acetate extracts from *Cetrelia* LFF. A common absorbance peak was detected in all extracts except *Cetrelia chicitae* (RT = 16 min).

perties against *P. aeruginosa* at 103.44 µg/mL. In this study, applied concentrations of LFF extracts had no effect on bacterial growth. Furthermore, several studies demonstrate antioxidant and other biological activities of *Cetrelia* genus. However, the number of QS inhibition studies on lichen substances are limited (Fernández-Moriano et al., 2015; Shrestha, 2015; Yamamoto et al., 2015; Gokalsin et al., 2019). This study presents the QSI potential of lichen metabolites that *Cetrelia* LFF contain.

Industrial scale production of LFFs present a serious challenge for now. Therefore, the amount of extracts one can obtain may vary. Moreover, the solubility of the crude extracts also changes according to lichen species. Due to limited amount of extracts and their solubilities, concentrations of each sample are different in this study. However, the concentrations of bioactive metabolites that cause the QS inhibition in crude extracts can also vary due to multiple parameters such as species metabolism and environmental conditions. Therefore, isolation and analysis of pure compounds in future studies would highlight the QS inhibition properties of LFFs more efficiently. This study intends to show that LFF cultures can also be utilized to produce QSI compounds instead of collecting slow growing lichen thalli from nature. With further research, it is possible to identify and isolate active compounds from the extracts and assess their compatibility for drug research.

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