JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

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Inhibition of *Pseudomonas aeruginosa* and *Escherichia coli* O157:H7 Biofilm Formation by Plant Metabolite ε-Viniferin

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Supporting Information

ABSTRACT: Pathogenic biofilms are associated with persistent infection due to their high resistances to diverse antibiotics. *Pseudomonas aeruginosa* infects plants, animals, and humans and is a major cause of nosocomial diseases in patients with cystic fibrosis. In the present study, the antibiofilm abilities of 522 plant extracts against *P. aeruginosa* PA14 were examined. Three *Carex* plant extracts at a concentration of 200 μg/mL inhibited *P. aeruginosa* biofilm formation by >80% without affecting planktonic cell growth. In the most active extract of *Carex pumila*, resveratrol dimer ε-viniferin was one of the main antibiofilm compounds against *P. aeruginosa*. Interestingly, ε-viniferin at 10 μg/mL inhibited biofilm formation of enterohemorrhagic *Escherichia coli* O157:H7 by 98%. Although *Carex* extracts and *trans*-resveratrol are known to possess antimicrobial activity, this study is the first to report that *C. pumila* extract and ε-viniferin have antibiofilm activity against *P. aeruginosa* and *E. coli* O157:H7.

KEYWORDS: *biofilm, Carex plant, Escherichia coli O157:H7, Pseudomonas aeruginosa, ε-viniferin*

INTRODUCTION

Bacteria coexist in multispecies communities and can infect insects, plants, animals, and humans. In nature, most bacteria are likely to form surface-attached biofilm communities as a survival strategy.[1] On the other hand, biofilms are of considerable medical importance as they account for >80% of microbial infections.[2] Furthermore, pathogenic biofilms pose a challenge because they have enhanced resistance to conventional antibiotics, host defenses, and external stresses and, thus, are difficult to control in medical and industrial settings.[3−5] Currently, an explosive amount of biofilm research is being conducted to discover novel compounds capable of inhibiting biofilms without allowing bacteria to develop drug resistance.

Pseudomonas aeruginosa is the most common Gram-negative bacterium found in nosocomial and life-threatening infections in cystic fibrosis patients,[6] and to date, dozens of antibiofilm compounds to this bacterium have been identified from diverse natural sources. Major antibiofilm compounds identified in plants include brominated furanones,[7] garlic,[8] ursine triterpenes,[9] corosolic acid and asiatic acid,[10] ginseng,[11] and 3-indolylacetonitrile.[12] In addition, a few plant extract libraries have been used to control *P. aeruginosa* biofilm formation.[13,14]

P. aeruginosa PA14 is a clinical isolate obtained from a burn patient, and it is also a potent foliar pathogen in a variety of plants.[15,16] In the present study, 522 medicinal plant extracts were screened for the ability to inhibit *P. aeruginosa* PA14 biofilm formation without affecting cell growth. In addition, we attempted to identify active antibiofilm compounds in *Carex* extracts that efficiently inhibit *P. aeruginosa* PA14 biofilm formation. Furthermore, the effect of a novel antibiofilm compound ε-viniferin on enterohemorrhagic *Escherichia coli* O157:H7 was investigated.

MATERIALS AND METHODS

Plant Extracts. The 522 Asian medicinal plant extract library used was obtained from the Korean Plant Extract Bank (http://extract.pdrc.re.kr/extract/f.htm, Daejeon, Republic of Korea). The plant library was publically available, and a list of the plants investigated is provided in Supplementary Table 1 of the Supporting Information with vendor IDs. The plants tested were selected for reasons of diversity and possible medicinal activity as determined by literature searches. The extraction procedure used was as previously described.[17] Briefly, plants were dried at room temperature for 5 days away from direct sunlight and then ground, extracted with 99.8% methanol at 50 °C, and vacuum-dried at 45 °C. Methanol extracts were then aliquoted at 20 mg and stored at 4 °C until required. All dried plant extracts were dissolved in dimethyl sulfoxide (DMSO).

Chemicals. Thirty-three plant compounds, namely, 6-aminoflavone, apigenin, betulinic acid, biphenyl, caffeic acid, catechol, chrysin, *p*-coumaric acid, curcumin, daidzein, diphenylmethane, *trans*-ferulic acid, fisetine, flavone, genistein, hydroquinone, 4-hydroxybenzoic acid, 6-hydroxyflavone, kaempferol, luteolin, oxyresveratrol, phloretin, phloroglucinol, quercetin, resorcinol, *trans*-resveratrol, shikimic acid, sinapic acid, *cis*-stilbene, *trans*-stilbene, syringic acid, tannic acid, and vanillic acid, were purchased from Sigma-Aldrich (St. Louis, MO, USA). ε-Viniferin was obtained from the Korea Chemical Bank (http://www.chembank.org, Daejeon, Republic of Korea) and had been originally purified from the seed extract of *Paeonia lactiflora* (Paeoniaceae). The detailed purification procedure used and physicochemical and spectroscopic data, including NMR spectra, of ε-viniferin have been previously described.[18]

Bacterial Strains and Growth Rate Measurements. *P. aeruginosa* PA14,[19] *P. aeruginosa* PAO1,[20] and *E. coli* O157:H7 (ATCC43895) were used. All experiments were conducted in Luria−

Received: March 4, 2013
Revised: July 2, 2013
Accepted: July 2, 2013
Published: July 2, 2013

dx.doi.org/10.1021/jf4009313 | J. Agric. Food Chem. 2013, 61, 7120−7126

Bertani (LB) medium at 37 °C. Bacteria were initially streaked from −80 °C glycerol stock onto a LB plate, and a fresh single colony was inoculated into LB (25 mL) medium in 250 mL flasks and cultured at 37 °C and 250 rpm. Overnight cultures were reinoculated into medium at a dilution of 1:100. Cell growths were determined by measuring optical densities at 600 nm using a spectrophotometer (UV-160, Shimadzu, Japan). Each experiment was performed using at least two independent cultures.

Biofilm Assay and Antibiofilm Screening. A static biofilm formation assay was performed in 96-well polystyrene plates (SPL Life Sciences, Korea), as previously described.[21] Briefly, cells were inoculated into LB medium (total volume = 300 μL) at an initial turbidity of 0.05 at 600 nm and then cultured with or without plant extracts for 24 h without shaking. Biofilms in 96-well plates were stained with crystal violet and dissolved in 95% ethanol, and absorbances were measured at 570 nm (OD_{570}) to quantify total biofilm formation. Cell growths in 96-well plates were also measured at 620 nm (OD_{620}). Initial antibiofilm screening was performed using plant extracts at 0.2 mg/mL in four wells for two independent cultures. For more detailed analysis, results were averaged from at least 12 replicate wells.

Confocal Laser Microscopy. Using a confocal laser microscopy (Nikon eclipse Ti, Tokyo, Japan) and *P. aeruginosa* PAO1/pMRP9-1 and *E. coli* O157:H7/pCM18 tagging a green fluorescent protein, the static biofilm with or without *trans*-resveratrol and ε-viniferin in the 96-well plates was visualized by excitation with an Ar laser 488 nm (emission, 500−550 nm) and a 20× objective. Color confocal images were made using NIS-Elements C version 3.2 (Nikon eclipse). For each experiment, at least 10 random positions of three independent cultures were chosen for microscopic analysis.

HPLC Assays for *trans*-Resveratrol and ε-Viniferin. Concentrations of *trans*-resveratrol and ε-viniferin were measured by reverse-phase HPLC using a 4.6 × 250 mm Zorbax Eclipse XDB-C18 column (Agilent Technology, Santa Clara, CA, USA) and acetonitrile/water gradient (10% acetonitrile increasing to 20% over 5 min, to 50% at 35 min, and finally to 100% at 40 min).[22] The flow rate used was 1.0 mL/min. Plant extracts, *trans*-resveratrol (Sigma-Aldrich), and purified ε-viniferin were dissolved in methanol and filtered through a 0.2 μm syringe filter prior to injection. HPLC peaks of *trans*-resveratrol and ε-viniferin were identified using retention times and by comparing UV−vis spectra with standards. Under these conditions, the retention times and absorbance maxima of *trans*-resveratrol and purified ε-viniferin were 19.5 min (306 nm) and 25.3 min (324 nm), respectively.

Virulence Factor Assays of *P. aeruginosa*. Overnight *P. aeruginosa* PA14 cultures were diluted 1:100 in LB medium and then treated with *Carex pumila* extract (0.1 mg/mL), ε-viniferin (50 μg/mL), or DMSO as a control. The pyocyanin assay was adapted;[23] *P. aeruginosa* was grown for 7 h, and culture supernatants were extracted with chloroform and analyzed spectrophotometrically. The rhamnolipid assay was adapted;[24] *P. aeruginosa* was grown for 7 h, and culture supernatants were assayed using the orcinol colorimetric assay. The pyochelin assay was adapted;[25] *P. aeruginosa* was grown for 7 h, and culture supernatants were assayed using the nitrite−molybdate reagent. At least two independent experiments were conducted.

RESULTS AND DISCUSSION

Antibiofilm Activities of *Carex* Extracts. To identify antibiofilm compounds, methanol extracts from a total of 522 plants (331 genera, 481 species, including 20 different plant parts) were screened in 96-well plates. For this screening, 0.2 mg/mL of each plant extract was used to minimize antimicrobial effects. In fact, no growth reduction of *P. aeruginosa* PA14 cells >50% of final cell density was observed for any plant extract. Detailed information on *P. aeruginosa* PA14 growth and biofilm formation in the presence of the 522 plant extracts is provided in Supporting Table 1 of the Supporting Information.

The 522 plant extracts controlled *P. aeruginosa* PA14 biofilm formation with widely different efficiencies (Figure 1).

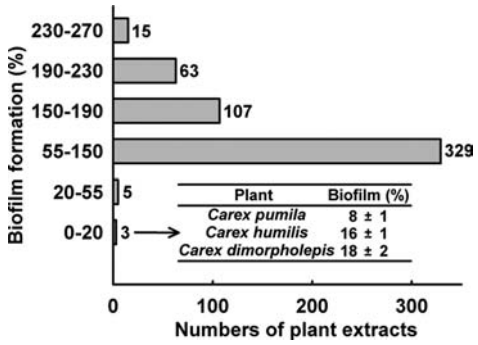

Figure 1. Histogram of *P. aeruginosa* biofilm formation in the presence of the 522 plant extracts. Biofilm screening for *P. aeruginosa* PA14 was performed using an extract concentration of 0.2 mg/mL in 96-well plates over 24 h at 37 °C. Numbers beside bars indicate numbers of plant extracts. Biofilm formation (%) on the *Y*-axis represents changes in biofilm formation, that is, biofilm formation in the presence of plant extract/biofilm formation by the untreated control × 100. Detailed information on biofilm formation and cell growth is provided in Supporting Table 1 in the Supporting Information.

Generally, more plant extracts increased than inhibited *P. aeruginosa* PA14 biofilm formation. Initially, *Carex dimorpholepis* was found to have antibiofilm activity, and 16 *Carex* species were added to the investigation. Of the 522 plants, extracts of three *Carex* species, namely, *C. pumila*, *Carex humilis*, and *C. dimorpholepis*, inhibited *P. aeruginosa* PA14 by >80%. Another five *Carex* species inhibited *P. aeruginosa* PA14 biofilm formation by >45% (Figure 1 and Supporting Table 1 in the Supporting Information). The most active was *C. pumila* extract, which dose-dependently inhibited biofilm formation (Figure 2A). More specifically, at 0.1 mg/mL this extract inhibited *P. aeruginosa* PA14 biofilm formation by 89% (Figure 2A).

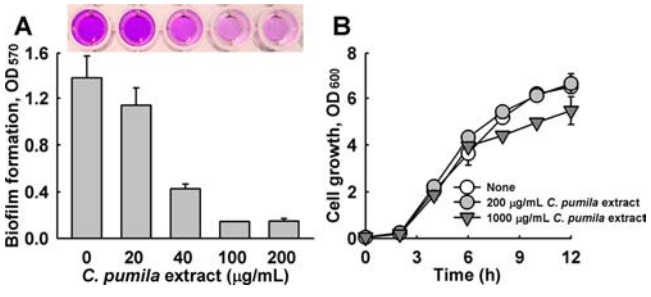

Figure 2. Biofilm reductions achieved by *C. pumila* extract. Biofilm formation (OD_{570}) of *P. aeruginosa* PA14 was quantified in the presence of *C. pumila* extract after 24 h in 96-well plates (A). Planktonic cell growths of *P. aeruginosa* PA14 in the presence of *C. pumila* extract (200 or 1000 μg/mL) were measured at 600 nm in 250 mL flasks agitated at 250 rpm (B).

The cell growth of *P. aeruginosa* PA14 was investigated to identify antibiofilm compounds without antimicrobial activity. The presence of *C. pumila* extract at concentrations up to 1 mg/mL did not diminish *P. aeruginosa* PA14 cell growth (Figure 2B). It is important to note that reduced biofilm formation by *C. pumila* extract was due to its antibiofilm activity

dx.doi.org/10.1021/jf4009313 | J. Agric. Food Chem. 2013, 61, 7120−7126

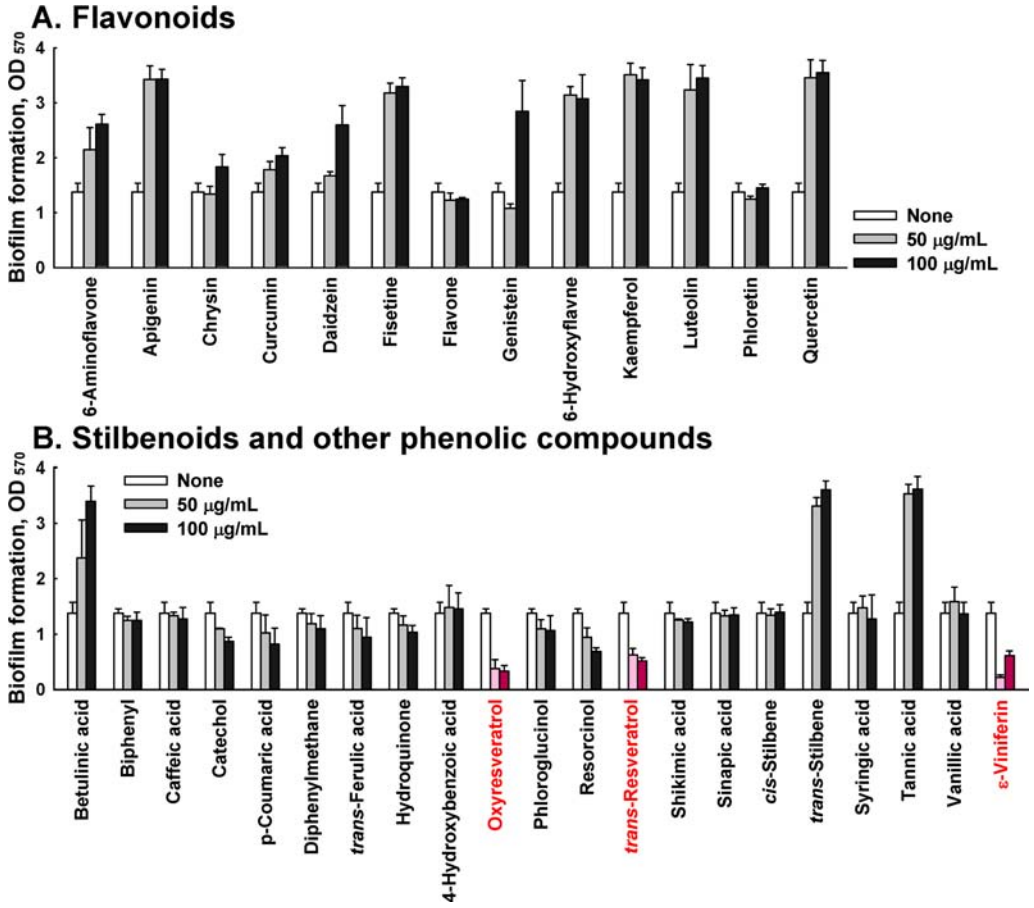

Figure 3. Effects of *Carex* metabolites on *P. aeruginosa* biofilm formation. Biofilm formations (OD_{570}) of *P. aeruginosa* PA14 were quantified in the presence of selected *Carex* metabolites, such as flavonoids (A) and stilbenoids and other phenolic compounds (B), after 24 h in 96-well plates without shaking. At least two independent experiments were conducted (total of 12 wells). Error bars indicate standard deviations.

and not due to its antimicrobial activity, which suggests that *C. pumila* extract might not lead to drug resistance.

Effect of *C. pumila* Metabolites on *P. aeruginosa* Biofilm Formation. *Carex* plants are characterized by the production of diverse polyphenols, including stilbene derivatives, lignans, and flavonoids.[26−28] Of the various *Carex* and other plant metabolites, 33 commercial compounds and 1 purified compound (*ε*-viniferin) were selected and assayed for antibiofilm activity against *P. aeruginosa* PA14.

Interestingly, of these 34 compounds, 9 flavonoids and betulinic acid, *trans*-stilbene, and tannic acid significantly increased *P. aeruginosa* biofilm formation at a concentration of 100 *μ*g/mL (Figure 3). In fact, many common plant metabolites were found to enhance *P. aeruginosa* biofilm formation, which is in line with our finding that 185 plant extracts increased *P. aeruginosa* biofilm formation by >50% (Figure 1). From the ecological perspective, it is likely that *P. aeruginosa* has developed a defense system against plant source agents that allows it to form more biofilms, which is similar to *P. aeruginosa* inducing its biofilm formation in the presence of subinhibitory concentrations of aminoglycoside antibiotics.[4]

Inhibitions of *P. aeruginosa* Biofilm Formation by *trans*-Resveratrol and *ε*-Viniferin in *C. pumila* Extract. The most noticeable inhibitions of *P. aeruginosa* biofilm formation were achieved by oxyresveratrol, *trans*-resveratrol, and *ε*-viniferin (Figure 3B). Initially, *trans*-resveratrol showed antibiofilm activity and then the resveratrol dimer *ε*-viniferin and oxyresveratrol were investigated because the extract of *C. pumila* was found to contain *ε*-viniferin,[28] which was originally purified from the seed extract of *Paeonia lactiflora* for this study.[18]

The presence of *trans*-resveratrol and *ε*-viniferin in *C. pumila* extract was confirmed by HPLC, which showed that standard *trans*-resveratrol and *ε*-viniferin matched corresponding peaks and UV spectra of components (Figure 4A), whereas oxyresveratrol was not detected in *C. pumila* extract. The concentrations of *trans*-resveratrol and *ε*-viniferin in *C. pumila* extract were 0.30 and 19.7 mg/g, respectively,; thus, *ε*-viniferin was found to be a major stilbene in this extract. Similarly, *C. humilis* contained 0.31 mg/g *trans*-resveratrol and 5.95 mg/g *ε*-viniferin, respectively.

Further biofilm experiments showed that *trans*-resveratrol and *ε*-viniferin dose-dependently inhibited the biofilm formation of two *P. aeruginosa* strains, PAO1 and PA14 (Figure 4B,C). Specifically, *trans*-resveratrol at 50 *μ*g/mL inhibited *P. aeruginosa* PAO1 biofilm formation by 92%, and *ε*-viniferin at 50 *μ*g/mL inhibited *P. aeruginosa* PA14 biofilm formation by 82% without affecting planktonic cell growth. Fifty percent biofilm inhibitory concentrations of the *C. pumila* extract and *ε*-viniferin against *P. aeruginosa* PA14 are 31 and 16 *μ*g/mL, respectively. Using a confocal microscopy, the biofilm inhibition of *P. aeruginosa* was confirmed (Figure 4D). Because *C. pumila* extract contained much more *ε*-viniferin than *trans*-resveratrol (Figure 4A), we believe that *ε*-viniferin is largely responsible for the antibiofilm activity of the *C. pumila* extract.

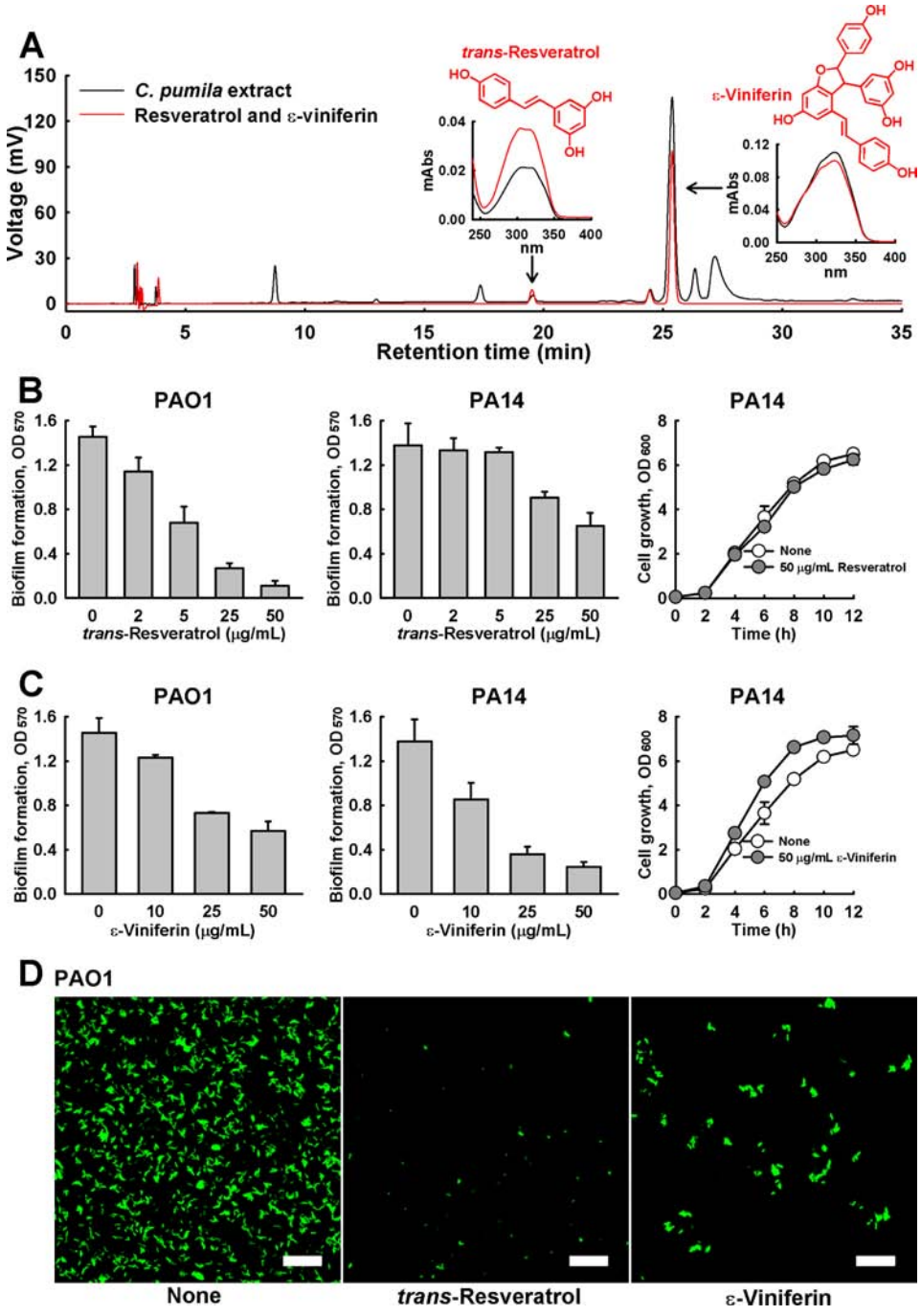

Figure 4. (A) Effect of *trans*-resveratrol and *ε*-viniferin on *P. aeruginosa* biofilm formation; HPLC chromatogram of *C. pumila* extract. Chemical structures and UV−visible spectra of *trans*-resveratrol and *ε*-viniferin are shown as insets. The standard samples indicated by the red line contained commercial *trans*-resveratrol (0.5 *μ*g/mL) and purified *ε*-viniferin (10 *μ*g/mL). (B, C) Inhibitory effects of *trans*-resveratrol (B) and *ε*-viniferin (C) on biofilm formation of two *P. aeruginosa* strains, PAO1 and PA14; planktonic cell growths of *P. aeruginosa* PA14 in the presence of *trans*-resveratrol and *ε*-viniferin. (D) Biofilm observation by a confocal laser microscopy; biofilm formation of *P. aeruginosa* PAO1/pMRP9-1 tagging a green fluorescent protein in the 96-well plates with or without *trans*-resveratrol (50 *μ*g/mL) and *ε*-viniferin (50 *μ*g/mL). Scale bar represents 20 *μ*m. The experiment was performed in triplicate.

Inhibitions of *E. coli* O157:H7 Biofilm Formation by *C. pumila* Extract and *ε*-Viniferin. The effects of *C. pumila* extract and *ε*-viniferin were also investigated on the biofilm formation of another pathogenic bacterium, *E. coli* O157:H7. *C. pumila* extract and purified *ε*-viniferin extract both dose-dependently inhibited *E. coli* O157:H7 biofilm formation (Figure 5). In particular, *ε*-viniferin at 10 *μ*g/mL inhibited *E. coli* O157:H7 biofilm formation by 98% without affecting planktonic growth. The biofilm inhibition of *E. coli* O157:H7 was confirmed by confocal microscopy (Figure 5C). The present study is the first to report that *C. pumila* extract and *ε*-viniferin have antibiofilm activity against *P. aeruginosa* and *E. coli* O157:H7.

Effect of *C. pumila* Extract and *ε*-Viniferin on the Production of *P. aeruginosa* Virulence Factors. Because *P. aeruginosa* produces diverse virulence factors controlled by

dx.doi.org/10.1021/jf4009313 | J. Agric. Food Chem. 2013, 61, 7120−7126

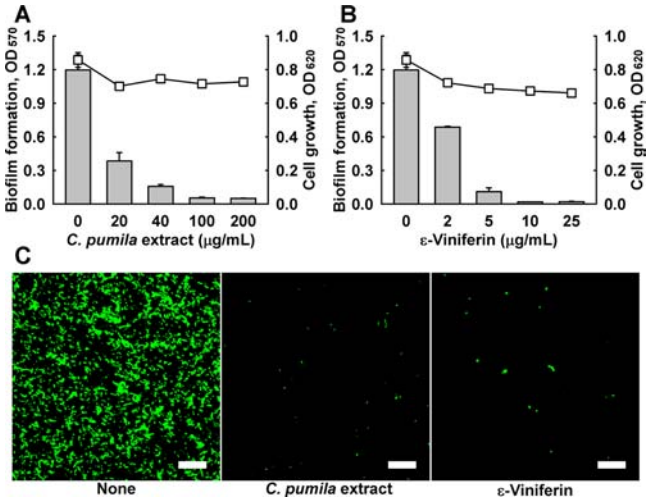

Figure 5. Effects of *C. pumila* extract and ε-viniferin on *E. coli* O157:H7 biofilm formation. Biofilm formation (OD_{570}) of *E. coli* O157:H7 was quantified in the presence of *C. pumila* extract (A) and ε-viniferin (B) after 24 h in 96-well plates. Cell growths of *E. coli* O157:H7 in the presence of *C. pumila* extract and ε-viniferin were measured at 620 nm in 96-well plates. (C) Biofilm formation of *E. coli* O157:H7/pCM18 tagging a green fluorescent protein in the 96-well plates with or without *C. pumila* extract (100 μg/mL) and ε-viniferin (10 μg/mL). Scale bar represents 20 μm.

quorum sensing, which also controls its biofilm development,[7,29,30] we investigated the productions of pyocyanin, rhamnolipid, and pyochelin by *P. aeruginosa* in the presence of ε-viniferin or *C. pumila* extract. Only pyocyanin production was slightly reduced by *C. pumila* extract and by ε-viniferin (Supporting Information, Supporting Figure 1). To understand the molecular mechanism of *C. pumila* extract and ε-viniferin, further study is required. Because flavonoid-rich orange extract[31] and polyphenols including *trans*-resveratrol[32] inhibited quorum sensing signals in *Chromobacterium violaceum*, it is interesting to investigate the effect of *C. pumila* extract and ε-viniferin on *P. aeruginosa* quorum sensing.

***Carex* Species, Resveratrol Derivatives, and Biofilm Control.** In the Cyperaceae family, the genus *Carex*, which includes sedges, contains as many as 2000 species worldwide.[33] Only a few *Carex* species have been previously investigated, and these studies have revealed the presence of resveratrol oligomers and other stilbene derivatives, which have attracted attention due to their nutraceutical potentials.[26,34] *trans*-Resveratrol is abundant in red wine and has been reported to have antibacterial, antiaging, anticarcinogenic, anti-inflammatory, and antioxidant properties in humans.[35,36] ε-Viniferin, a dimer of resveratrol, is found in grapevines (*Vitis* species)[37] and *Carex* plants[28] and has been reported to have fungicidal, antioxidant, hepatoprotective, and P450 inhibitory activities.[37,38] Interestingly, in the present study, only 8 *Carex* plant extracts of 522 plant extracts inhibited *P. aeruginosa* biofilm formation (Figure 1). On the other hand, extracts of *Vitis amurensis*, *Vitis coignetiae*, and *Vitis vinifera* increased *P. aeruginosa* biofilm formation (Supporting Information, Supporting Table 1). Furthermore, the addition of several red wines containing *trans*-resveratrol[39] did not reduce *P. aeruginosa* biofilm formation (data not shown). These results suggest that unlike *Carex* extracts, *Vitis* extracts and red wines may contain large amounts of biofilm-enhancing compounds, such as flavonoids and tannic acid (Figure 3).

Recently, it was reported that resveratrol at 3.2 mg/mL inhibits the biofilm formation of Gram-positive *Propionibacterium acnes* without antimicrobial activity[40] and that resveratrol and its derivatives reduce *E. coli* O157:H7 adhesion to epithelial cells.[41] In the present study, *trans*-resveratrol and ε-viniferin from *C. pumila* were both found to have antibiofilm activity against *P. aeruginosa* without antimicrobial activity. Furthermore, ε-viniferin exhibited antibiofilm activity against *E. coli* O157:H7, infections of which are associated with an elevated risk of hemolytic-uremic syndrome when antibiotics are administered.[42] Unlike most antibiotics that primarily aim to inhibit cell growth, ε-viniferin did not affect cell growth and, thus, offers the possibility of reducing the risk of antibiotic resistance.[43,44]

Plants and bacteria have developed advanced defense mechanisms. This study demonstrates that various plant extracts contain biofilm enhancers and inhibitors against *P. aeruginosa* (Figures 1 and 3). Here, we provide comprehensive data regarding the effects of various plant extracts/compounds on *P. aeruginosa* biofilm formation. Furthermore, we report for the first time that ε-viniferin in *C. pumila* extract and *P. lactiflora* extract acts as a biofilm inhibitor.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Supporting Figure 1 and Supporting Table 1. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

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Funding

This research was supported by the Bioindustry Technology Development Program, Ministry for Food, Agriculture, Forestry and Fisheries, Republic of Korea, and a World Class University Grant (R32-2008-000-20082-0) of the National Research Foundation of Korea.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

The ε-viniferin used in this study was kindly provided by Korea Chemical Bank of Korea Research Institute of Chemical Technology.

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