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주 의
(편집순서 8)

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자생식물추출이용 항생제내성세균의 병원성억제물질 발굴

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발간등록번호
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자생식물추출물 이용 항생제내성 세균의 병원성 억제물질 발굴

(Discovery of anti-virulence compounds from natural plant extracts against antibiotic-resistant pathogens)

영남대학교

농 림 축 산 식 품 부

주 의

1. 이 보고서는 농림축산식품부에서 시행한 신진연구자 사업의 연구보고서입니다.

2. 이 보고서 내용을 발표할 때에는 반드시 농림축산식품부에서 시행한 신진연구자 사업의 연구결과임을 밝혀야 합니다.

3. 국가과학기술 기밀유지에 필요한 내용은 대외적으로 발표 또는 공개하여서는 아니 됩니다.

제 출 문

농림축산식품부 장관 귀하

이 보고서를 “자생식물추출물 이용 항생제내성 세균의 병원성 억제물질 발굴”의 보고서로 제출합니다.

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요 약 문

Ⅰ. 제 목

자생식물추출물 이용 항생제내성 세균의 병원성 억제물질 발굴

Ⅱ. 연구개발의 목적 및 필요성

항생제 남용은 세균들의 항생제 내성을 유발하여 세균 감염 만성질환 관련 세계적인 이슈가 되고 있음. 이에 세균의 성장을 억제하고 죽이는데 목적인 항생제와는 달리 항병원성 물질은 세균의 성장에는 큰 영향을 미치지 않으면서 병원성만을 억제하므로 일반 항생제보다 세균의 항병원성 물질에 대한 저항성이 적게 유발됨. 항병원성 물질의 중요한 예로는 항생제 내성을 유발하는 생물막형성 억제제와 또한 세포성장에는 영향을 미치지 않으면서 병독소의 생성을 억제하는 경우가 있음. 따라서 본 연구에서는 다양한 자생식물추출물 library를 이용하여 생물막 형성 억제제이면서 병독소 생성 억제제를 스크리닝하고 이렇게 발견한 물질의 작용 기작을 연구하고자 함.

Ⅲ. 연구개발 내용 및 범위

자생식물추출물 500 종류 이상을 이용하여 세 병원성 균주의 생물막 억제제에 대한 스크리닝 후 이차적으로 병독소 생성 억제 물질을 스크리닝함. 스크리닝 중 세균성장 억제 (항생효과가 있는) 물질은 제외하며 추가적으로 스크리닝된 물질들의 항생제내성에 미치는 영향과 병독소 생성 억제력 실험을 수행함.

Ⅳ. 연구개발결과

1. 항생제내성 녹농균의 생물막형성과 용혈 작용을 분석하여 사초 (Carex) 추출물에서 새로운 생물막 억제제인 ε-viniferin 발견함. ε-Viniferin 이 장출혈대장균의 생물막형성을 억제함을 규명함. 국제저명학술지 논문 게재

2. 항생제내성 황색포도상구균의 병독소인 staphyloxanthin 생성과 적혈구 용혈을 억제 효과가 가장 뛰어난 flavone을 발견함. qRT-PCR를 활용하여 flavone의 작용기작을 밝힘. 국제저명학술지 논문 게재

Ⅴ. 연구성과 및 성과활용 계획

ε-viniferin과 flavone이 새로운 항생제 대체 물질로서 사용 가능함. 현재 동물세포와 동물을 이용한 실험 중임.

SUMMARY

Ⅰ. Title

Discovery of anti-virulence compounds from natural plant extracts against antibiotic-resistant pathogens

Ⅱ. Purpose of this project

Emergence of antibiotic-resistant bacteria is a urgent problem worldwide. Therefore, a non-antibiotic strategy is required, and this could be provided by anti-virulence compounds that target bacterial virulence rather than cell viability, which may be less prone to develop drug resistance. As important anti-virulence approaches are anti-biofilm strategy and anti-toxin strategy without affect bacterial cell growth. Therefore, objectives of this study are to discover novel anti-virulence compounds from natural plant extracts against antibiotic-resistant pathogens and to understand their action mechanism.

Ⅲ. Research scopes.

Using more than 500 plant extracts, we wanted to identify novel anti-biofilm against three pathogenic bacteria (*Escherichia coli* O157:H7, *Pseudomonas aeruginosa* PAO1, and *Staphylococcus aureus*) without affect bacterial cell growth. Additionally, anti-toxin ability has been investigated.

Ⅳ. Results of this project.

1. Against antibiotic-resistant *Pseudomonas aeruginosa* and *Escherichia coli* O157:H7, we found novel anti-biofilm compound ε-viniferin from *Carex pumila* extract. Published an SCI paper.
2. Against antibiotic-resistant *Staphylococcus aureus*, we found novel anti-toxin compound flavone that inhibited staphyloxanthin production and blood hemolysis. Action mechanism of flavone has been revealed via qRT-PCR. Published an SCI paper.

Ⅴ. Expected research outputs.

Both ε-viniferin and flavone can be used as an alternative of antibiotic approach. Currently, tests with animal cell and animals are on going before clinical trial.

CONTENTS

목 차

제 1 장 연구개발과제의 개요

1.1. 연구과제의 배경과 필요성

□ 최근 항생제내성 세균의 출현은 만성감염 환자 및 면역관련 환자들의 치사율을 크게 높이고 특히 병원내 항생제내성 세균감염은 모든 환자들에게 큰 위협임. 항생재내성 관련 여러 기작 중 세균의 생물막 형성은 항생제 내성을 크게 증가시킴.

□ 항생제 내성을 극복할 미생물 항병원성 (anti-virulence) 물질 개발의 필요성. 다양한 항생제가 발견되었음에도 불구하고 여전히 많은 병원성 세균은 인류의 건강을 위협하고 있음. 특히 항생제의 역할이 세균의 성장을 저해하고 죽이는 데 목적이 있어 항생제 남용은 세균들의 항생제 내성을 유발함. 하지만 현재 새로이 개발되는 항생제의 수는 매우 제한적임 (전 세계적으로 연간 2-3개). 이에 새로운 형태의 항균물질인 항병원성 물질의 연구가 절실히 필요함.

□ 항병원성 물질은 항생제와는 달리 세균의 성장에는 영향을 미치지 않고 병원성 물질 생산을 저해하는 것. 그러므로 일반 항생제보다 세균이 항병원성 물질에 대한 저항성을 적게 유발하는 것으로 알려져 있음. 항병원성 물질의 주요한 예로는 세균간의 의사소통 (quorum sensing 또는 정족수인식)을 방지하여 병원성을 감소시키는 정족수인식 억제제와 항생제 내성을 유발하는 생물막 (biofilm)의 형성을 억제하는 물질, 또는 병독소 생성 억제제 등이 있음

치석 상처에 생물막 의료기기에 생물막

그림 1. 미생물 생물막의 종류

□ 병원성 미생물을 포함한 대부분의 (95% 이상) 미생물들은 생물막이라는 자기보호막을 형성함으로써 자신을 외부의 여러 환경인자로부터 보호하려 함. 가장 잘 알려진 생물막의 예로는 치석을 들 수 있으며 또한 상처부위에 세균들이 생물막을 형성하여 상처의 회복속도를 늦추고 상처의 흉터를 크게 남김.

□ 더욱이 여러 병원성 미생물 (*Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Eshcherichia coli* 등)의 생물막 형성은 면역력이 저하된 환자들의 치사율을 크게 높임. 또한 카테터 (catheter), 각종 삽입 보형물 (implant), 그리고 인공장기 같은 의료기구에도 생물막 형성이 문제를 야기함. 특히 생물막을 형성한 세균들은 부유 상태 세균들보다 1,000배 이상의 항생제 내성을 가질 수 있음. 그러므로 항생제 내성을 갖지 않는 생물막 형성 저해제 (억제제)의 탐색은 전 세계적인 관심의 대상임.

그림 2. 생물막형성 세균의 항생제 내성 모형

□ 세균감염은 직접적으로 폐렴, 욕창, 충치, 상처감염 등에 치명적이지만 이차적으로 세균이 혈액 속에 번식할 때 패혈증을 유발함. 패혈증은 만성질환환자 (폐렴, 당뇨병, 류마티스) 및 면역질환자에게 위협적임. 특히 패혈증의 발병균은 *S. aureus*, *P. aeruginosa*, *E. coli* 등인데 항생제 내성을 가진 세균인 경우 생명에 크게 위협적이므로 항독소 및 항용혈성 치료제의 개발 또한 시급함

제 2 장 국내외 기술개발 현황

□ 미생물의 생물막 형성은 다양한 무생물표면과 생물체표면에서 발견되고 있으며, 최근 생물막의 생성 조절이 의학, 생물공학, 환경공학, 식품공학 분야에서 매우 중요하게 인식되고 있음. 생물막 연구의 중요성은 PubMed에서 생물막 (biofilm)을 주요 주제 (key word)로 검색하면 2011년 4,000개 이상의 논문이 발표되었음. 특히 생물막형성이 항생제 내성뿐만 아니라 병원성에 밀접한 관련이 있다고 알려지고 있어 다양한 분야에서 연구가 폭발적임.

□ 의학 및 생명공학적인 측면에서만 아래와 같은 국내 여러 연구팀에서 새로운 생물막 억제제의 탐색과 그 작용기작을 밝히려는 연구를 활발히 진행하고 있음. 연세대 윤상선 교수팀과 김백일 교수팀, 고려대 김세헌 교수팀, 박우준 교수팀, 류지훈 교수팀, 서울대 윤제용 교수팀, 부산대 박성훈 교수팀, 건국대학

교 권지향 교수. 하지만 국내에선 항병원성 물질 탐색은 아직 보고되지 않고 있으며, 생물막 억제제 탐색을 위한 library의 규모가 아직 매우 제한적임.

□ 생물막에 관한 전 세계적 관심과 연구에 비하여 어떤 물질들이 생물막을 억제하고 병원성을 저해하는가에 대한 연구 결과는 그리 많지 않음. 지난 10년간 밝혀진 주요 생물막 형성 억제제를 살펴보면 아래의 표 1과 같음. 여러 반응성 화합물 또는 단백질을 이용한 생물막 형성 억제 및 무기물이나 표면처리를 통한 생물막 억제는 이 표에 포함시키지 않음.

<표 1 생물막 억제제 및 항병원성물질들>

생물막 억제제 또는 항병원성 물질	유래	특징
furanone과 furanone 유사체	열대 해조류 *Delisea pulchra* 또는 합성	세포간 교신 억제 독성이 있으며 불안정함
4-nitro-pyidine-N-oxide	마늘 추출물	세포간 교신 억제
bromoageliferin	해면 (marine sponge)	세포간 교신 억제
cis-2-decenoic acid	*P. aeruginosa* 유래	생물막 억제제
ursolic acid	열대 ebony 나무	황대사를 통해 작용
ellagic acid, esculetin, fisetin	녹차추출물	*In silico* 스크리닝에 의해 발견
D-아미노산들	미생물 대사물	L-아미노산들은 영향 없음
nitric oxide	미생물 대사물	c-di-GMP 조절
LED209	유기합성물	정족수인식억제 및 병원성억제
Virstatin	유기합성물	병독소 생성 억제
halogenated anthranilic acid	미생물 유래 유도체	정족수인식억제 및 병원성억제
oroidine	유기합성물	생물막 분산
fluorouracil	uracil 유도체, 합성	DNA 복제를 억제
indole과 hydroxyindole	미생물 대사물	미생물에 의해 분해 단점
3-indolylacetonitrile	십자화속 식물유래	생물막과 병독소 생성 억제
phloretin	사과에 다량 함유	생물막 억제 및 항염증 효과

□ 위 표에서도 알 수 있듯이 많은 식물유래 추출물과 미생물 유래 천연물이 생물막 형성을 억제함이 확인됨. 특히 최근 D-아미노산, LED209 와 Virstatin은 병원성 세균의 생물막형성 억제와 항병원성 물질로서 최근 각각 Science 저널에 보고되며, 현재 호주의 Kjelleberg교수와 덴마크의 Givskov교수를 중심으로 정족수인식 억제제인 열대 해조류유래 푸라논 유도체를 녹농균의 항병원성물질로 사용하기 위한 상업적 컨소시움이 활발히 진행되고 있으며, 미국의 Costerton, Greenberg, Kolter, Rahme 교수를 중심으로 발견된 항병원

성 물질의 상업화가 경쟁적임.

제 3 장 연구개발수행 내용 및 결과

□ 자생식물 추출물로는 현재 한국생명공학연구원 내 보유중인 자생식물추출물 500 종류이상과 한국한방산업진흥원에 보유중인 정제 생약천연물 50종류이상을 분양받아 일차 스크리닝으로 중요 병원성 세균인 *S. aureus*와 *P. aeruginosa,* 장출혈성 *E. coli* O157:H7의 생물막 형성 억제제에 대한 스크리닝을 수행함. 스크리닝은 96-well plate를 이용하여 세균의 세포성장에는 영향을 미치지 않으면서 생물막 형성을 억제하는 물질을 발굴함.

□ 사용하는 세 가지 병원성 세균에 대해 간단히 언급하면, *S. aureus* (포도상구균)는 가장 잘 알려진 병원유래 감염 세균으로써 장기간의 항생제 남용으로 인해 여러 가지 항생제에 내성을 갖는 multidrug-resistant *Staphylococcus aureus* (superbug로 알려짐)의 출현을 전 세계적으로 유발하고 일반적으로 적혈구 용해를 유발.

□ *P. aeruginosa* (녹농균)은 폐렴이나 cystic fibrosis 환자의 치사율에 큰 영향을 미치는 세균으로 항생제 내성을 유발하고 생물막연구에 가장 많이 활용되는 균주이며 적혈구용해 유발.

□ *E. coli* O157:H7 (장출혈성 대장균)은 후진국뿐만 아니라 선진국에서도 여전히 문제 (변이종이 2011년 유럽을 강타함)가 되는 인체 병원성 세균으로 감염특징은 점막부착과 동시에 점막파괴를 야기하여 면역력이 저하된 환자, 영유아 및 노약자의 생명을 위협함. 현재 신장에서의 적혈구 용혈을 방지할 수 있는 효과적인 처방이 없는 실정.

□ 생물막형성 억제제 스크리닝은 본 연구자가 지난 5년간 수행해 온 실험으로 먼저 96-well plate를 이용하여 적당량의 식물 추출물 (항생 효과가 매우 적은 200 μg/mL 정도농도)을 병원성 배양액에 첨가하여 세균의 생물막 형성 억제를 조사함. 확인을 위해 연속실험 장치인 flow-cell에서 결과를 확인함. 더욱이 인간대장상피세포를 이용하여 세균의 부착능을 조사할 수 있음.

그림 3. 생물막 형성 실험. (A) 96-well plate 이용 스크리닝 (초기 실험에 사용), (B) 연속실험장치에서 생물막 형성의 3-D 영상을 confocal 현미경으로 관찰 가능. (C) 인간 대장상피세포에 병원성 세균 (GFP을 발현하는)의 부착능을 조사.

□ 일차 스크리닝으로 얻은 생물막 형성 억제제에 대해 이차적으로 세균의 여러 병원성 (병독소 화합물과 단백질) 생성 억제 특성을 조사하고 또한 세균에 의한 용혈에 미치는 영향을 조사. 특히, *P. aeruginosa*(녹농균)의 주요 병독소인 푸른빛 pyocyanin과 *S. aureus*의 주요 병독소인 노랑빛 staphyloxanthin은 색깔을 띄므로 스크리닝에 매우 유리한 장점이 있음

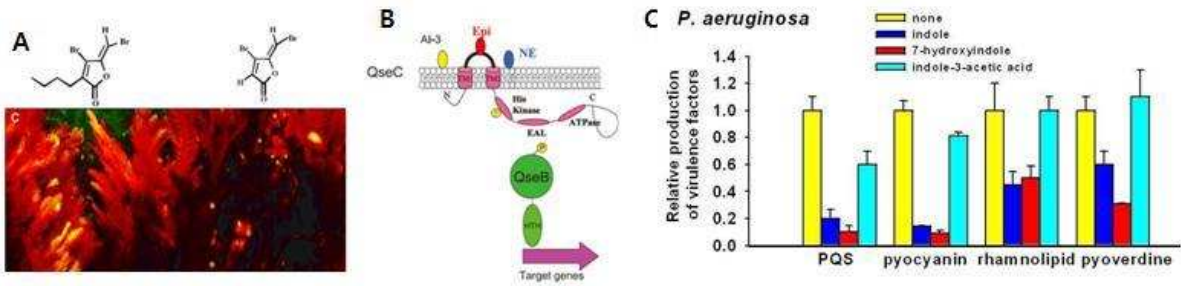

그림 4. 항병원성물질의 예. A) 적조류의 furanone이 녹농균의 정족수인식 억제 (EMBOJ, 22: 3803-3815, 2003), B) 유기합성물인 LED209의 장출혈대장균의 정족수인식시스템 억제 (Science 321: 1078-1080, 2008), C) 본 책임연구자가 밝힌 미생물 유래 인돌과 7-hydroxyindole이 녹농균의 정족수인식을 억제하여 다양한 병독소 생성을 억제 (Microbial Biotech, 2: 75-90, 2009)

연구결과 1: 자생식물 사초 (Carex) 추출물에서 새로운 생물막 억제제인 ε-viniferin 발견

초록

병원성 생물막은 높은 항생제 내성을 유발하여 세균 감염에 큰 문제이다. 녹농균 (*Pseudomonas aeruginosa*) 식물, 동물 및 인체에 유해한 세균으로, 특히 병원에서 주로 감염되며 cystic fibrosis 환자에게 매우 유해하다. 본 연구에서는 522종의 자생 식물 추출물의 항생물막 능력을 녹농균 PA14 대상으로 측정하였다. 좀보리사초 추출물 (200 μg/mL)이 녹농균 생물막을 80% 이상 억제하였다. 좀보리사초 추출물에서 활성물질 탐색을 통해 가장 활성이 있는 resveratrol dimer ε-viniferin을 발견 하였다. 또한 ε-viniferin (10 μg/mL) 장출혈 대장균의 (*Escherichia coli* O157:H7) 생물막 형서을 98% 이상 억제하였다. 비록 resveratrol이 항생 효과가 있다고 알려져 있지만, 본 연구를 통해서 ε-viniferin 이 두 종류의 병원성 균주 (녹농균과 장출혈 대장균)에 대한 항생물 효과가 있음을 최초로 보고하였다.

연구 배경

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 more than 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]

Pseudomonas 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 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.

재료 및 실험 방법

식물추출물. 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 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 ^{o}C, and vacuum-dried at 45 ^{o}C. Methanol extracts were then aliquoted at 20 mg and stored at 4 ^{o}C until required. All dried plant extracts were dissolved in dimethyl sulfoxide (DMSO).

화합물. 33 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-hydroxyflavne, 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, 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]

사용세균 및 성장. *P. aeruginosa* PA14,[19] *P. aeruginosa* PAO1,[20] and *E. coli* O157:H7 (ATCC43895) were used. All experiments were conducted in Luria-Bertani (LB) medium at 37 ^{o}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 re-inoculated 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.

생물막 실험 및 스크리닝. 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})toquantifytotalbiofilmformation.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 twelve replicate wells.

Confocal 레이저 현미경. 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 to 550 nm) and a 20 x 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 분석. Concentrations of *trans*-resveratrol and ε-viniferin were measured by reverse-phase HPLC using a 4.6 x 250 mm ZORBAX Eclipse XDB-C18 column (Agilent Technology, Santa Clara, 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/visible 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.

녹농균에 대한 병독소 실험. Overnight *P. aeruginosa* PA14 cultures were diluted 1:100 in LB medium and then treated with *C. 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.

실험결과 및 고찰

사초 추출물의 항생물막 효과. 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 greater than 50% of final cell density was observed for any plant extract.

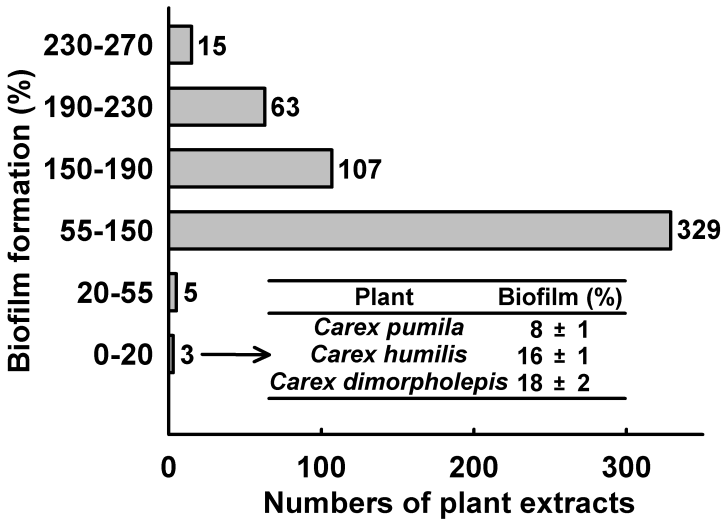

Fig. 1. Histogram of *P. aeruginosa* biofilm formation in the presence of the 522 plant extracts. Biofilm screening for *P. aeruginosa* PA14 was performed using an extract concentration of 0.2 mg/mL in 96-well plates over 24 h at 37 °C. Numbers on the tops of bars indicate numbers of plant extracts. 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 x 100. Detailed information on biofilm formation and cell growth is provided in Supporting Table 1.

The 522 plant extracts controlled *P. aeruginosa* PA14 biofilm formation with widely different efficiencies (Fig. 1). 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, *Carex pumila*, *Carex humilis,* and *C. dimorpholepis,* inhibited *P. aeruginosa* PA14 by more than 80%. Another five *Carex* species inhibited *P. aeruginosa* PA14 biofilm formation by more than 45% (Fig. 1 and Supporting Table 1). The most active was *C. pumila* extract, which dose-dependently inhibited biofilm formation (Fig. 2A). More specifically, at 0.1 mg/mL this

extract inhibited *P. aeruginosa* PA14 biofilm formation by 89% (Fig. 2A).

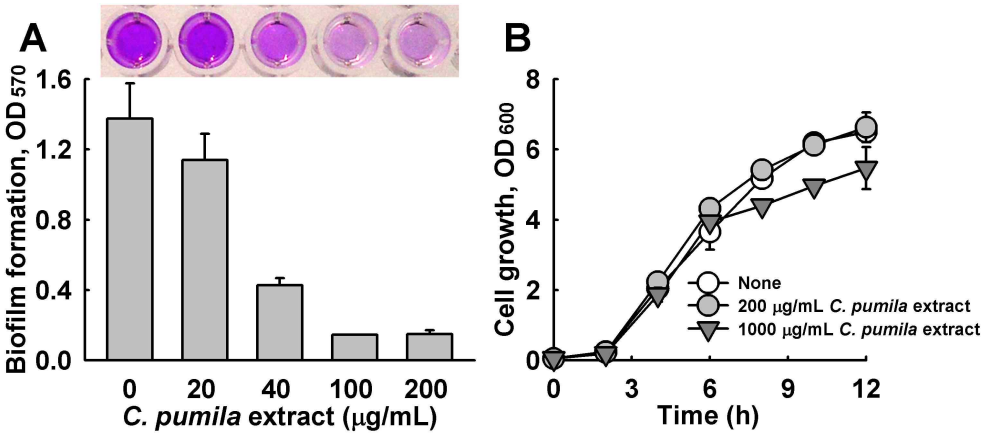

Fig. 2. Biofilm reductions achieved by *C. pumila* extract. Biofilm formation (OD_{570})of*P. aeruginosa* PA14 was quantified in the presence of *C. pumila* extract after 24 h in 96-well plates (A). Planktonic cell growths of *P. aeruginosa* PA14 in the presence of *C. pumila* extract (200 or 1000 μg/mL) 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 (Fig. 2B). It is important to note that reduced biofilm formation by *C. pumila* extract was due to its antibiofilm activity and not due to its antimicrobial activity, which suggests that *C. pumila* extract might not lead to drug resistance.

좀보리사초 화합물의 항생물막 효과. *Carex* plants are characterized by the productions of diverse polyphenols, including stilbene derivatives, lignans, and flavonoids.[26-28] Of the various *Carex* and other plant metabolites, 33 commercial compounds and one purified compound (ε-viniferin) were selected and assayed for antibiofilm activity against *P. aeruginosa* PA14.

Interestingly, of these 34 compounds, nine flavonoids and betulinic acid, *trans*-stilbene, and tannic acid significantly increased *P. aeruginosa* biofilm formation at a concentration of 100 μg/mL (Fig. 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 more than 50% (Fig. 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 that *P. aeruginosa* induces its biofilm formation in the presence of sub-inhibitory concentrations of aminoglycoside antibiotics.[4]

trans-resveratrol과 ε-viniferin 항생물막 효과. The most noticeable inhibitions of *P. aeruginosa* biofilm formation were achieved by oxyresveratrol, *trans*-resveratrol, and ε-viniferin (Fig. 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]

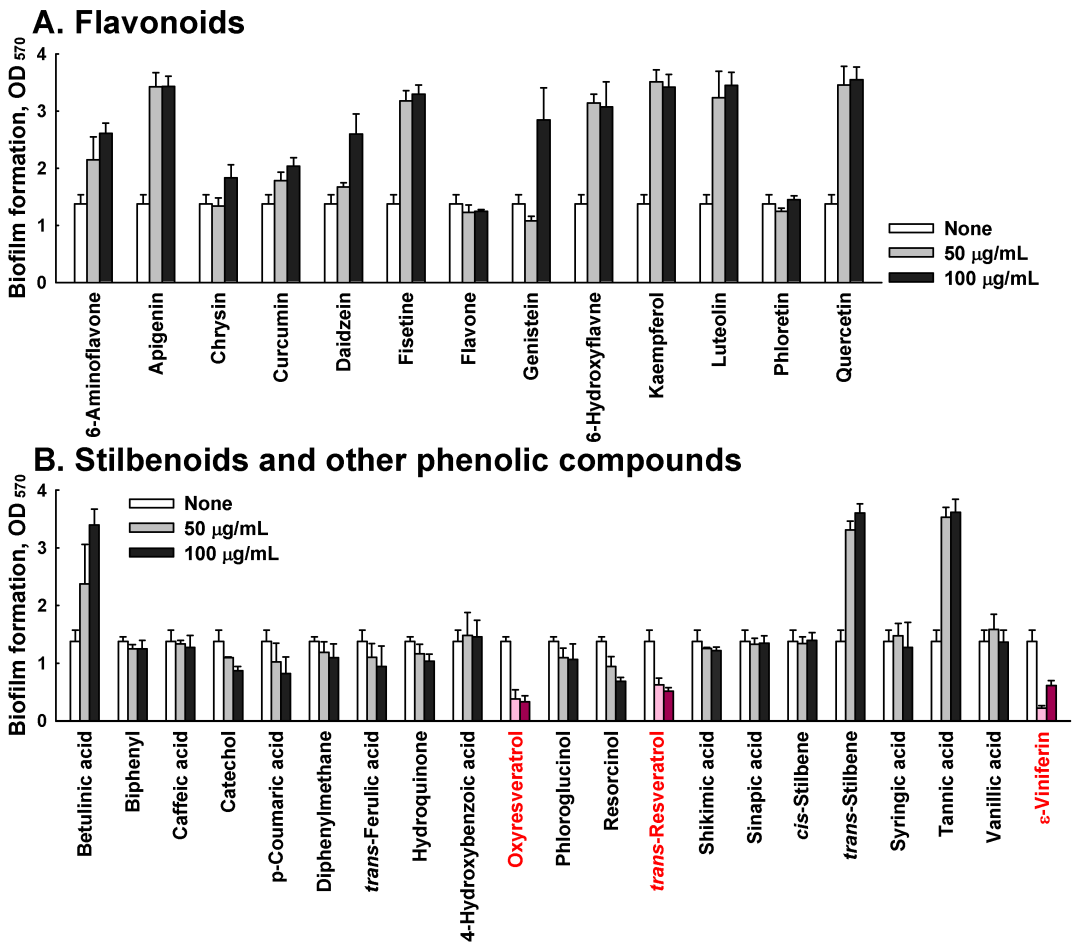

Fig. 3. Effects of *Carex* metabolites on *P. aeruginosa* biofilm formation. Biofilm formations (OD_{570})of*P. aeruginosa* PA14 were quantified in the presence of selected *Carex* metabolites, such as, flavonoids (A), stilbenoids, and other phenolic compounds (B), after 24 h in 96-well plates without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

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 (Fig. 4A), while oxyresveratrol was not detected in *C. pumila* extract. The concentrations of *trans*-resveratrol and ε-viniferin in *C. pumila* extract were 0.30 mg/g and 19.7 mg/g, respectively, thus, ε-viniferin was found to be a major stilbene in this extract. Similarly, *Carex humilis* contained 0.31 mg/g of *trans*-resveratrol and 5.95 mg/g of 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 (Fig. 4B and 4C). Specifically, *trans*-resveratrol at 50 μg/mL inhibited *P. aeruginosa* PAO1 biofilm formation by 92%, and ε-viniferin at 50 μg/mL inhibited *P. aeruginosa* PA14 biofilm formation by 82% without affecting planktonic cell growth. 50% biofilm inhibitory concentrations of the *C. pumila* extract and ε-viniferin against *P. aeruginosa* PA14 are 31 μg/ml and 16 μg/ml, respectively. Using a confocal microscopy, the biofilm inhibition of *P. aeruginosa* was confirmed (Fig. 4D). Because *C. pumila* extract contained much more ε-viniferin than *trans*-resveratrol (Fig. 4A), we believe that ε-viniferin is largely responsible for the antibiofilm activity of *C. pumila* extract.

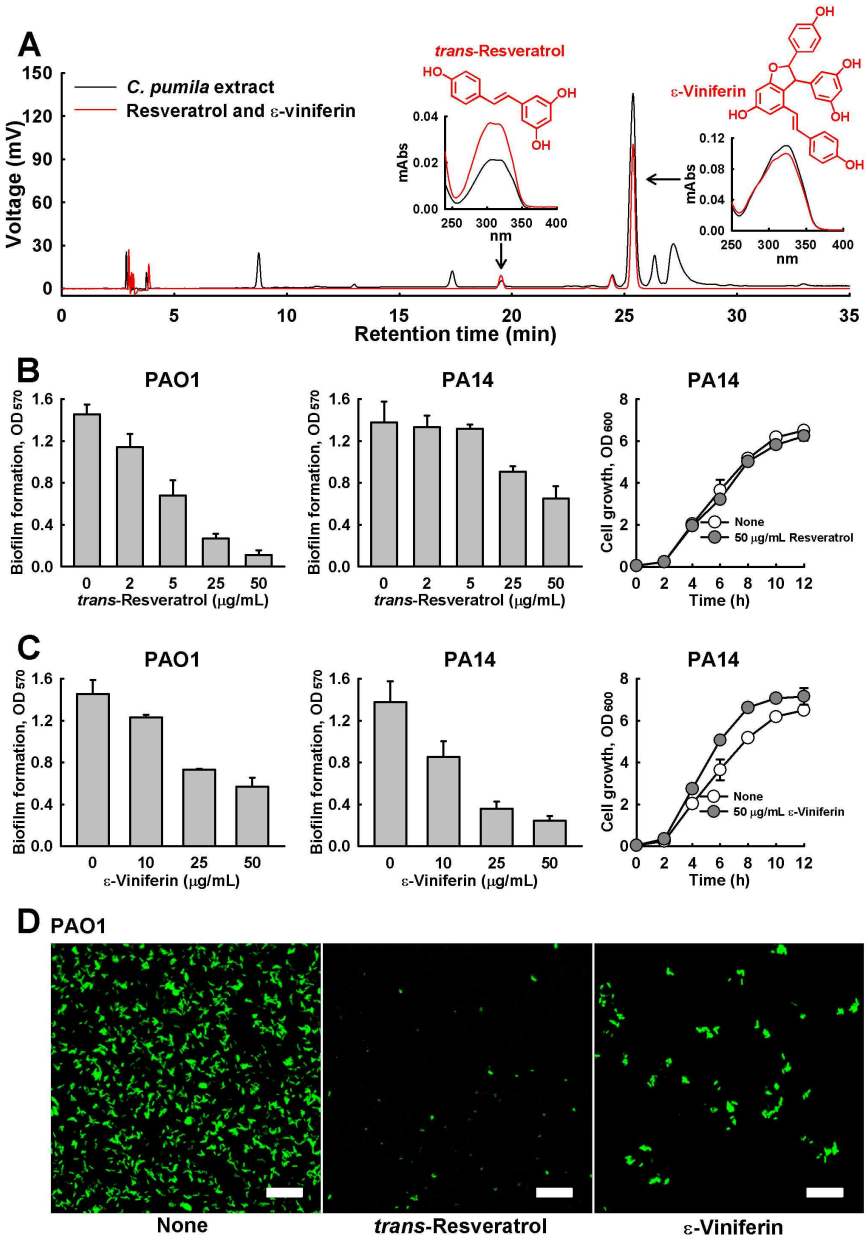

Fig. 4. Effect of *trans*-resveratrol and ε-viniferin on *P. aeruginosa* biofilm formation. HPLC chromatogram of *C. pumila* extract. Chemical structures and UV/visible spectra of *trans*-resveratrol and ε-viniferin are shown as insets (A). The standard samples were indicated by the red line contained commercial *trans*-resveratrol (0.5 μg/mL) and purified ε-viniferin (10 μg/mL). The 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. Biofilm observation by a confocal laser microscopy (D). Biofilm formation of *P. aeruginosa* PAO1/pMRP9-1

tagging a green fluorescent protein in the 96-well plates with or without *trans*-resveratrol (50 μg/mL) and ε-viniferin (50 μg/mL). Scale bar represents 20 μm.

좀보리사초와 ε-viniferin. 의 장출혈 대장균에 대한 항생물막 효과. 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 (Fig. 5). In particular, ε-viniferin at 10 μg/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 a confocal microscopy, (Fig. 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.

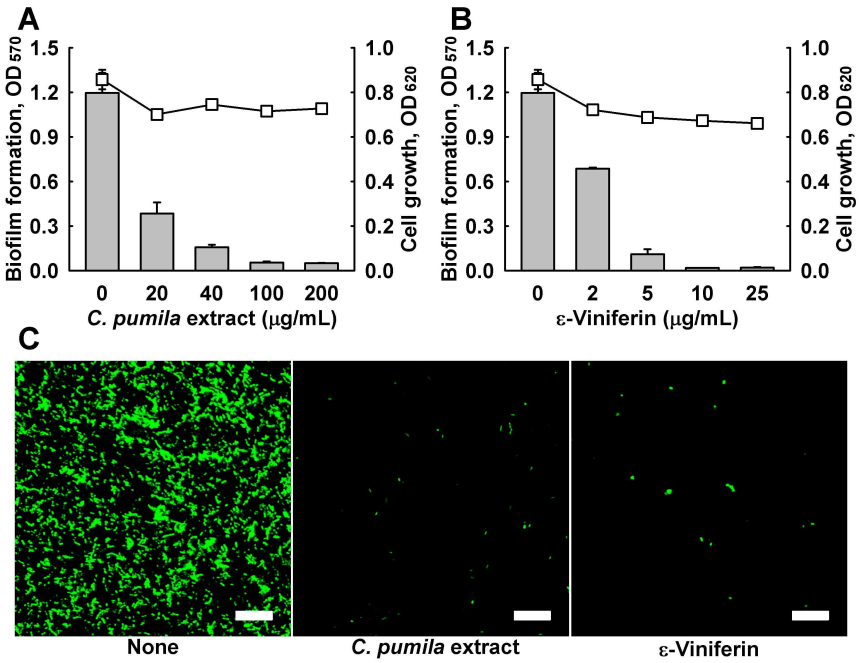

Fig. 5. Effects of *C. pumila* extract and ε-viniferin on *E. coli* O157:H7 biofilm formation. Biofilm formation (OD_{570})of*E. coli* O157:H7 was quantified in the presence of *C. pumila* extract (A) and ε-viniferin (B) after 24 h in 96-well plates. Cell growths of *E. coli* O157:H7 in the presence of *C. pumila* extract and ε-viniferin were measured at 620 nm in 96-well plates. 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) (C). Scale bar represents 20 μm.

좀보리사초와 ε-viniferin. 의 녹농균의 병독소 억제 효과. Since *P. aeruginosa* produces diverse virulence factors controlled by 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 Fig. 1). To understand the molecular mechanism of *C. pumila* extract and ε-viniferin, further study is required. Because flavonoids-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.

In Cyperaceae family, the genus *Carex,* which includes sedges, contains as many as 2,000 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 has attracted attention due to their nutraceutical potentials.[26,34] *trans*-Resveratrol is abundant in red wine and has been reported to have anti-bacterial, anti-aging, anti-carcinogenic, anti-inflammatory, and anti-oxidant 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, anti-oxidant, hepatoprotective, and P450 inhibitory activities.[37,38] Interestingly, in the present study, only eight *Carex* plant extracts out of 522 plant extracts inhibited *P. aeruginosa* biofilm formation (Fig. 1). On the other hand, extracts of *Vitis amurensis*, *Vitis coignetiae*, and *Vitis vinifera* increased *P. aeruginosa* biofilm formation (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 (Fig. 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 resveratrol and its derivatives reduces *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* (Fig. 1 and Fig. 3). Here, we provide comprehensive data regarding the effects of various plant extracts/compounds on *P. aeruginosa* biofilm formation. Furthermore, we reported for the first time that ε-viniferin in *C. pumila* extract and *P. lactiflora* extract acts as a biofilm inhibitor.

연구결과 2: 플라본 (flavone)의 황색포도상구균의 병독소 생성 억제효과

초록

황색포도상구균 (*Staphylococcus aureus*)은 항생제 내성을 쉽게 유발하고 병원에서 감염성이 높다. 특히 황색포도상구균은 다양한 외독소를 생성하는데 이로 인해 세균감염이 악화 되고 문제를 야기한다. 본 연구에서는 다양한 플라보노이들를 탐색하여 황색포도상구균의 병독소 생성을 억제하는 물질을 찾고자 하였다. 이를 통해 플라본 (flavone, 50 μg/mL)이 노랑색 면역회피물질인 staphyloxanthin 생성을 억제하였고, 또한 α-hemolysin을 억제하여 적혈구 용혈을 억제하였다. Staphyloxanthin 억제는 황색포도상구균이 과산화수소 존재하에서 100 배이상 민감함을 관찰하였다. 작용기작을 밝히기 위해 qRT-PCR을 수행하여 플라본이 α-hemolysin 생성 유전자인 *hla*과 global regulator 유전자인 *sae*을 현저히 억제함을 관찰하였다. 본 연구를 통하여 플라본이 항생제 내성을 쉽게 가지는 황색포도상구균 감염에 대한 병원성을 억제함을 보였다.

연구배경

Staphylococcus aureus is an important human pathogen that often exhibits antibiotic resistance and is responsible for worldwide outbreaks of nosocomial infections (13). This pathogen can secrete several exotoxins, such as hemolysin, enterotoxins, coagulase, TSST-1, and protein A, that are associated with specific diseases (15). *S. aureus* strains are also capable of producing the golden carotenoid pigment, staphyloxanthin that acts as a virulence factor, primarily being a bacterial antioxidant which protects the pathogen from the host's immune system in the form of reactive oxygen species (12).

Over several decades, numerous antibiotics have been developed and used for bacterial infections. However, there has been a significant decrease in the rate of discovery of new antibiotics (12). Furthermore, current usage of bactericidal compounds is often unsuccessful due to the emergence of

methicillin-resistant *S. aureus* (2, 11). Hence, unlike antibiotics that mostly aim to inhibit cell growth, alternative approaches such as antivirulence compounds have attracted strong research attraction. The antivirulence approach aims to reduce the production of virulence factors without affecting bacterial growth in order to impede the possible emergence of drug resistance (2, 6).

Major discoveries in the antivirulence approach against *S. aureus* include the inhibition of i) the virulence factor staphyloxanthin (12), ii) enterotoxins and hemolysins (23) iii) antibiotic resistant biofilm formation (1, 7, 8), and iv) bacterial quorum sensing (17). Recently, several plant compounds have been reported to decrease the virulence of *S. aureus* without affecting its growth. For example, thymol found in thyme (19) reduced enterotoxins and α-hemolysin production, luteolin (18) and chrysin (22) reduced α-hemolysin production, and fisetin (4) and olelic acid (21) inhibited the biofilm formation in *S. aureus*.

The overall goal of this study was to identify novel and potent antivirulence compounds from the screening of plant flavonoids against *S. aureus*. We investigated the effects of twelve flavonoids on the production of virulence factors, such as staphyloxanthin and α-hemolysin in *S. aureus*. Among the tested flavonoids, a subinhibitory concentration of flavone was identified as the most potent antivirulence compound without antimicrobial activity. This is the first report on the use of flavone to reduce the production of both staphyloxanthin and α-hemolysin of *S. aureus*.

재료 및 실험방법

미생물 및 화합물. All experiments were conducted at 37^{o}C,andtrypticase soy broth (TSB) was used for the culture of *S. aureus* (ATCC 25923) and *S. aureus* (ATCC 6538), which were obtained from the Korean Agricultural Culture Collection. Two *S. aureus* strains were used to reinforce our findings. Chemicals including twelve flavonoids (flavone (99%), 6-aminoflavone (97%), 6-hydroxyflavone (98%), apigenin (97%), chrysin (97%), curcumin (94%), daidzein (98%), fisetin (98%), genistein (98%), luteolin (98%), phloretin (99%), and quercetin (98%)) were purchased from Sigma-Aldrich Co. (Missouri, USA).

The structures of the flavonoids are shown (Fig. 1). All twelve flavonoids were dissolved in dimethyl sulfoxide (DMSO).

미생물 배양. *S. aureus* strains were initially streaked from -80°C glycerol stock on LB plates and a fresh single colony was inoculated in TSB (2 mL) in 14-mL tubes and cultured at 37°C and 250 rpm for all experiments except cell growth measurement. Overnight cultures were re-inoculated at 1:100 dilution in the medium. For cell growth measurements, a fresh single colony was inoculated in TSB (25 mL) in 250-mL flasks and cultured at 37°C and 250 rpm and optical densities were measured at 600 nm using a spectrophotometer (UV-160, Shimadzu, Japan). Each experiment was performed with at least two independent cultures.

Staphyloxanthin 분석. The bright golden coloration of this virulence factor facilitates the anti-virulence screening by the simple observation of color change (5). Also, a quantitative carotenoid assay method was adapted from the previous method (14). Briefly, cells were re-inoculated at 1:100 dilution in TSB medium and incubated for 16 hrs at 37°C with or without flavonoids. Cells (1 mL) were then collected by centrifugation at 13,000 rpm for 1 min and washed with 1 ml of phosphate-buffered saline (PBS). At this point, cell pellets were photographed to compare the staphyloxanthin production. For the extraction of carotenoid pigments, the cell pellets were resuspended in 0.2 mL of methanol by vortexing and this mixture was heated at 55°C for 3 min. Pigment extraction was separated from cell debris by centrifugation at 13,000 rpm for 10 min. This pigment extraction step was repeated 3 times and the optical densities of collected extractions were measured at 465 nm using a spectrophotometer (UV-160, Shimadzu, Japan). Each data point was averaged from at least three independent cultures.

과산화수소 저항성 실험. The resistance assay (survival test) with hydrogen peroxide was adapted (12). Overnight cultures grown for 16 h in TSB were re-grown to mid-log phase in TSB (turbidity at 600 nm of 1). Then, 0.1 mL of each culture was incubated with H_2O_2atafinalconcentrationof1.5% (v/v) for 60 min

with shaking at 250 rpm. The percentage of cells surviving the stresses was calculated as the number of colony-forming units (CFU)/mL remaining after each stress divided by the initial CFU/mL. Three independent experiments were conducted.

Table 1. Primer sequences for quantitative RT-PCR.

Gene	Name	Primer
hla	alpha-hemolysin	Forward 5'-CGG CAC ATT TGC ACC AAT AAG GC-3' Reverse 5'-GGT TTA GCC TGG CCT TCA GC-3'
sae	histidine protein kinase	Forward 5'-CGT ACA TTC AGA GTA GAA AAC TCT CGT AAT AC-3' Reverse 5'-GTT GCG CGA GTT CAT TAG CTA TAT AT-3'
agr	quorum-sensing regulator	Forward 5'-GTG AAA TTC GTA AGC ATG ACC CAG TTG-3' Reverse 5'-TGT AAG CGT GTA TGT GCA GTT TCT AAA C-3’
sigB	RNA polymerase sigma factor	Forward 5'-TCA CTG ATA GAA GGT GAA CGC TCT-3' Reverse 5'-AGT GAG CGA TGA ACT AAC CGC-3'
sar	biofilm regulator	Forward 5’-GAG TTG TTA TCA ATG GTC-3’ Reverse 5’-GTT TGC TTC AGT GAT TCG-3’
seo	enterotoxin O	Forward 5’-AGT CAA GTG TAG ACC CTA TT-3’ Reverse 5’-AGA TAT TCC ATC TAA CCA AT-3’
16s rRNA	a component of ribosomes	Forward 5'- TGT CGT GAG ATG TTG GG-3' Reverse 5'-CGA TTC CAG CTT CAT GT-3'

적혈구 용혈 실험 Hemolysis analysis was modified from the previous method (9). The lysis efficacy of human red blood cells was measured with whole cultures of *S. aureus* grown in the presence of flavonoids. Briefly, *S. aureus* cells were diluted at 1:100 with an overnight culture in TSB and cultured with or without all flavonoids at 50 μg/mL except luteolin at 25 μg/mL at 37°C for 16 h with shaking at 250 rpm. The cell cultures (50 μL including cells and culture supernatant) were added into diluted human red blood cells that had

previously been separated by centrifugation at 3,000 rpm for 5 min, washed with PBS buffer 3 times and diluted at 3% of red blood cells in PBS buffer. For hemolytic activity, the mixture was incubated at 37°C for 1 h with 250 rpm shaking. The supernatant was collected by centrifugation at 13,000 rpm for 10 min and the optical density was measured at 543 nm.

RNA isolation and real-time qRT-PCR. *S. aureus* (ATCC 25923) was cultivated in TSB with or without flavone for 16 h at 250 rpm. Before taking samples, RNase inhibitor (RNAlater, Ambion, TX, USA) was added and cells were immediately chilled for 30 s with dry ice and 95% ethanol (to prevent RNA degradation) before centrifugation at 13,000 g for 2 min. The cell pellets were immediately frozen with dry ice and stored at −80°C. Total RNA was isolated using a Qiagen RNeasy mini Kit (Valencia, CA, USA). To remove all DNA, the purified RNA was treated for 15 min with 30 Units of DNase I. To investigate the transcription of *hla* (α-hemolysin gene), *sae* (a global regulator), *agrA* (quorum-sensing gene), *sar* (accessory regulator A), *sigB* (RNA polymerase sigma factor), and *seo* (enterotoxin), they were quantified using qRT-PCR. The primer pairs for qRT-PCR are presented in Table 1. The 16s rRNA housekeeping gene was used. Real-time qRT-PCR was performed using the StepOneÔ Real-Time PCR system (Applied Biosystems, Foster City, CA) and SuperScript^{Ô} III PlatinumÒ SYBRÒ Green One-Step qRT-PCR kit (Invitrogen, Carlsbad, CA).

실험 결과

플라보노이드의 staphyloxanthin 생성 억제. To investigate the antivirulence activity against *S. aureus*, (ATCC 25923), twelve flavonoids at subinhibitory concentrations (10, 25, and 50 μg/mL) were screened for the reduction of staphyloxanthin production. The golden pigment staphyloxanthin could be visually identified in the cell pellets of *S. aureus*. Among the tested flavonoids, flavone, which is the backbone compound of flavonoids, most significantly reduced the staphyloxanthin production in *S. aureus* (Fig. 1). Quantitative analysis also clearly indicated that flavone reduced the staphyloxanthin production by 10-fold

compared to non- treatment control (data not shown). In the case of luteolin, its antimicrobial activity at 25 μg/mL (18) reduced the cell growth and staphyloxanthin production. The result of staphyloxanthin inhibition by flavone was similar to those in another *S. aureus* strain (ATCC 6538) (data not shown).

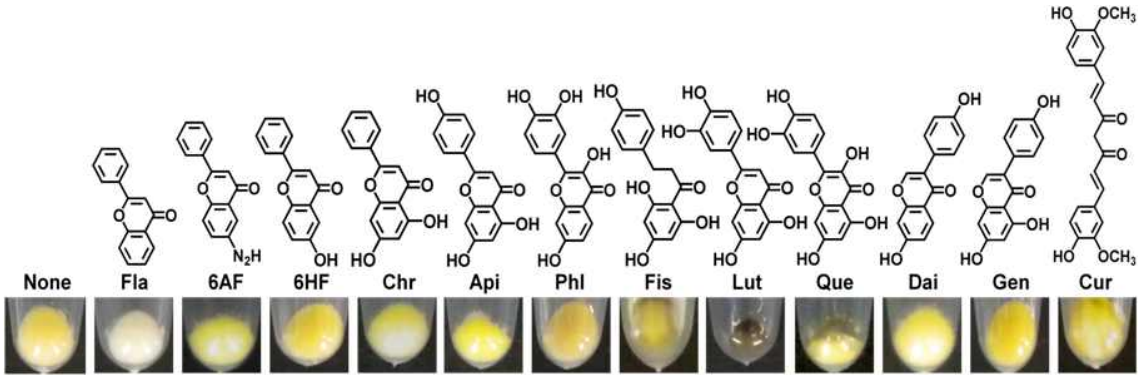

Fig. 1. Effect of flavonoids on the production of staphyloxanthin in *S. aureus*. *S. aureus* (ATCC 25923) was used. Cell pellets of 16-h grown *S. aureus* with or without flavonoids were measured for the production of staphyloxanthin. Fla: flavone; 6AF: 6-aminoflavone; 6HF: 6-hydroxyflavone; Chr: chrysin; Api: apigenin; Phl: phloretin; Fis: fisetin; Lut: luteolin; Que: quercetin; Dai: daidzein; Gen: genistein; Cur: curcumin. All flavonoids were used at 50 μg/mL, except luteolin, which was used at 25 μg/mL due to its antimicrobial activity. All of the compounds were dissolved in DMSO. DMSO was used as a control. The structures of the flavonoids are shown. The experiment was done in triplicate and representative images are shown.

플라본의 과산화수소 저항성 감쇠 Staphyloxanthin acts as an antioxidant by enabling the detoxification of host-immune system-generated ROS such as O^{2-} and hydrogen peroxide (H_2O_2)(12). Hence, we examined the effect of flavone on the survival rate of *S. aureus* in the presence of H_2O_2. As expected, flavone reduced H_2O_2 susceptibility by 100-fold, while structurally similar chrysin and 6-hydroxyflavone had no or much less effect on the survival rate(Fig.2).This result was attributed to the effect of flavone in reducing staphyloxanthin production in *S. aureus*.

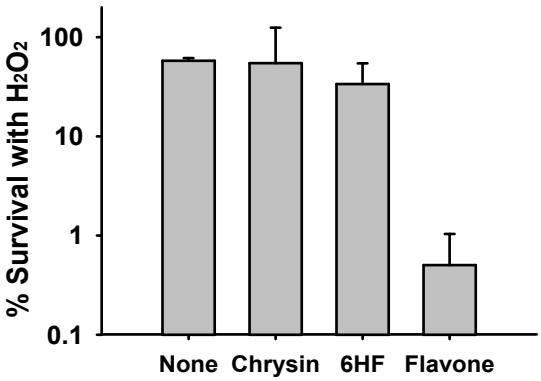

Fig. 2. Effect of flavonoids on hydrogen peroxide resistance. The survival of *S. aureus* (ATCC 25923) with or without flavonoids (chrysin, 6-hydroxyflavone (6HF), and flavone) was measured after H_2O_2(1.5%, v/v) treatment for 60 min. Flavonoids were used at 50 μg/mL. The percentage of survive cells was calculated as the number of colony forming units (CFU) per ml remaining after the H_2O_2stress divided by the initial CFU per ml. The experiment was performed in triplicate.

플라본의 적혈구용혈 감쇠 Since *S. aureus* can produce α-hemolysin, which is a pore-forming cytotoxin and causes hemolysis, we investigated the effect of flavonoids on blood hemolysis by *S. aureus*. Among the twelve tested flavonoids, eight showed a significant antihemolysis activity (Fig. 3). This result is consistent with that of the previous study (18) in that luteolin at subinhibitory concentration abolished the hemolysis activity of *S. aureus*. Moreover, seven more flavonoids, flavone, 6-aminoflavone, 6-hydroxyflavone, apigenin, phloretin, fisetin, and genistein, also markedly reduced hemolysis activity at their subinhibitory concentrations. Particularly, flavone clearly and dose-dependently inhibited the hemolytic activity of two *S. aureus* strains after 16 h culture (Fig. 4a and 4b). The reduction of hemolytic activity by flavone was similar at the different culture time points, such as 12 h and 24 h (data not shown).

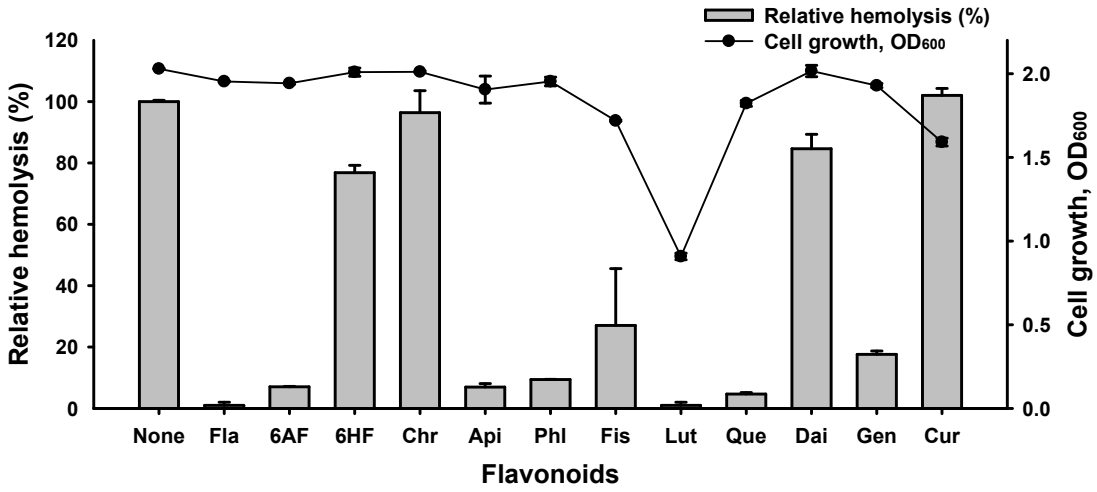

Fig. 3. Effect of flavonoids on hemolysis. The hemolysis screening was performed using human red blood cells upon adding *S. aureus* (ATCC 25923) cultures (50 μL) grown with flavonoids for 16 h. Fla: flavone; 6AF: 6-aminoflavone; 6HF: 6-hydroxyflavone; Chr: chrysin; Api: apigenin; Phl: phloretin; Fis: fisetin; Lut: luteolin; Que: quercetin; Dai: daidzein; Gen: genistein; Cur: curcumin. All flavonoids were used at 50 μg/mL, except luteolin, which was used at 25 μg/mL due to its antimicrobial activity.

A potential antivirulence compound without antimicrobial activity is preferred as this avoids the possible development of bacterial drug resistance. Thus, the toxicity of flavone was investigated by measuring the growth of planktonic *S. aureus* cells. Although flavone at 50 μg/mL slightly delayed the cell growth of two *S. aureus* strains, the growth was recovered at 14 h (Fig. 4c and 4d). As the optical density in the growth measurement, the numbers of viable cells were not significantly affected by flavone at 50 μg/mL (data not shown). The overall growth data indicated that the reduction of staphyloxanthin and antihemolysis activity of flavone was not due to its antimicrobial activity.

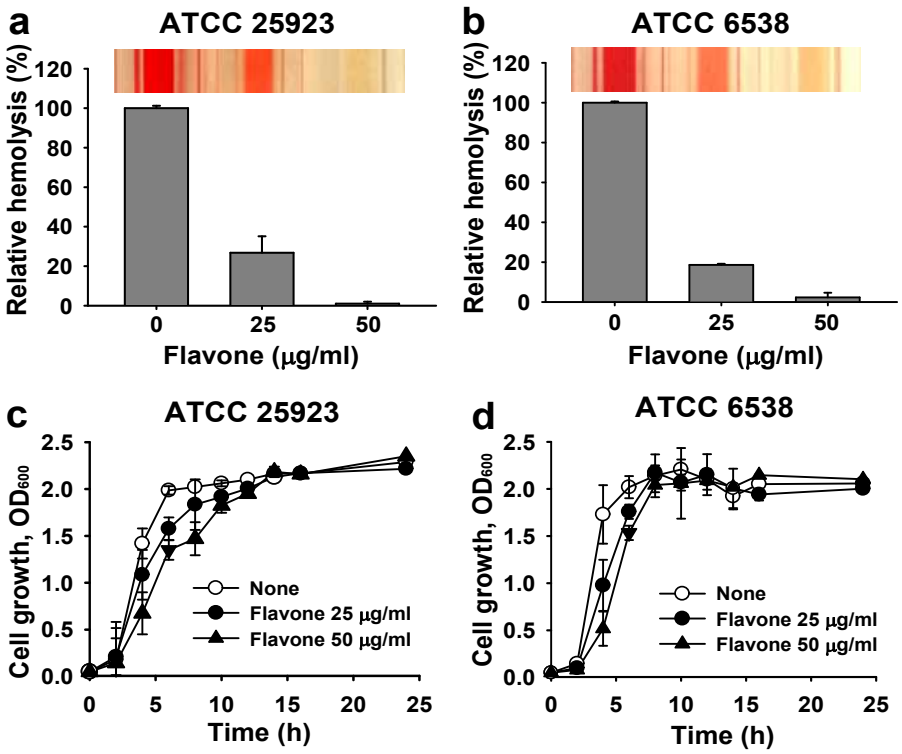

Fig. 4. Effect of flavone on hemolysis and cell growth in two *S. aureus* strains. The hemolysis assay was performed using human red blood cells upon adding two *S. aureus* (ATCC 25923 and ATCC 6538) cultures (50 μL) grown with flavone (0, 25, and 50 μg/mL) for 16 h. Picture of the spectrophotometer cuvettes are shown for the hemolysis activity. Planktonic cell growth of *S. aureus* was measured at 600 nm in 250 mL-flasks with 250 rpm.

플라본의 병독소 유전자 발현 감쇠 To investigate the mechanism of flavone's antivirulence activity, real-time qRT-PCR was used to determine a differential expression of virulence factor-related genes, such as *hla* (α-hemolysin gene), *sae* (a global regulator inducing *hla*(16), and *agrA* (quorum-sensing gene), in *S. aureus* cells with and without flavone. Flavone clearly repressed the transcription of *hla* by 11-fold and *sae* by 4-fold (Fig. 5), which supports the reduction of hemolysis in *S. aureus* cells by flavone (Fig. 4). However, flavone elevated *agrA* transcription by 4-fold and did not change the transcription of other virulence factor genes such as *sar*, *sigB* and *seo* (Fig. 5). The results support the previous

finding that the *agr* and *sae* might be inhibiting each other (16).

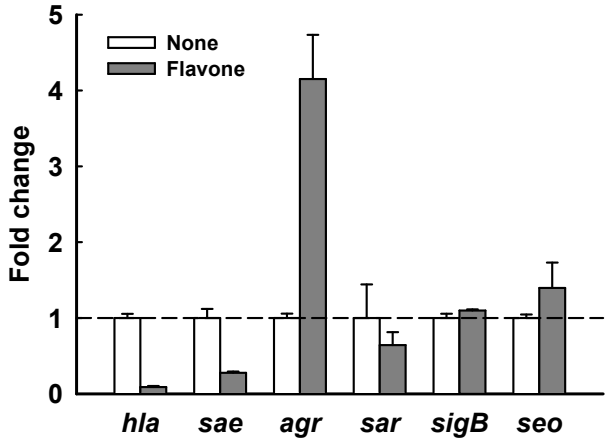

Fig. 5. Transcriptional profiles of *S. aureus* cells in the presence of flavone. Flavone was used at 50 μg/mL. Transcriptional profiles were measured by qRT-PCR. Fold change represents the change (*n*-fold) in transcription compared to the data in the absence of flavones (white bars, value of 1.0). The experiment was performed in duplicate.

고찰

In this study, we utilized dual screening to inhibit various virulence factors, such as staphyloxanthin and α-hemolysin, in *S. aureus*. Among twelve plant flavonoids, flavone reduced the production of staphyloxanthin, H_2O_2 resistance, and blood hemolysis without inhibiting the planktonic growth of *S. aureus*. This report is noteworthy as it is the first on the use of flavone to reduce both the hemolytic ability and staphyloxanthin production of *S. aureus* (Figs. 1, 3, and 4).

Flavonoids are ubiquitous in plants and are commonly found in fruit, vegetables, nuts, seeds, stems and flowers. They are biologically active in combating diseases in humans due to their diverse biological functions, such as antioxidative, antifungal, antiviral, antibacterial, and anticarcinogenic activity (3). Since the daily dietary intake of mixed flavonoids is estimated to be in the range of 500 – 1000 mg (20), they are likely to have minimal toxicity to humans (3), but further study is warranted to confirm this. Recently, the flavonoids luteolin (18) and chrysin (22) at subinhibitory concentrations showed

an ability to inhibit the hemolysis of *S. aureus*, and fisetin reduced the antibiotic-resistant biofilm formation in *S. aureus* (4), which demonstrated the potential antivirulence activity of these flavonoids. Compared to luteolin, chrysin, and fisetin, flavone specifically reduced the virulence factor of staphyloxanthin and the H_2O_2 resistance. Therefore, the present results have expanded the scope of previous studies and demonstrated that the functional groups of flavonoids differentially control several virulent phenotypes of *S. aureus*.

The expansion in bacterial resistance to antibiotics has created an urgent need for effective antimicrobial agents as well as antivirulence compounds against pathogenic bacteria. In this study, the dual screening of twelve flavonoids for two virulence factors was performed against *S. aureus*, and flavone demonstrated potential as a new potent antivirulence compound. Although the exact action mechanisms of flavone's antivirulence activity remains to be determined, the results suggest that the screening of a larger library of flavonoids will generate more potent therapeutics for the human pathogen *S. aureus*, and possibly for other pathogens as well. Recently, the flavonoid phloretin, which is abundant in apples, reduced the attachment of *Escherichia coli* O157:H7 to human colonic epithelial cells and also diminished colon inflammation in a rat model (10). Therefore, natural flavonoids are important sources for antivirulence compounds and flavone can be used as a basic structure in the design of potent antivirulence drugs.

제 4 장 목표달성도 및 관련분야에의 기여도

* 연도별 연구목표 및 평가착안점에 입각한 연구개발목표의 달성도 및 관련분야의 기술발전에의 기여도 등을 기술

구분	연도	연구개발의 목표	연구개발의 내용
1차년도	2012	최소 2종류 이상의 새로운 항병원성 물질을 발견하고 그 작용기작을 연구하여 2편의 국제전문학술지 (SCI급) 논문 발표와 1건 이상 국내특허를 출원하고자 함.	500종 이상의 다양한 자생식물 소재와 50종류이상의 한방소재 생약추출물을 이용하여 세 종류의 인체병원성 세균에 대한 생물막 형성 억제제이자 병원성 억제물질을 발굴하고자 함. 또한 활성물질의 분리, 규명과 더불어 그 작용기작을 분자적인 수준에서 연구함. 국제 저명학술지에 2편 논문 발표. 국제학술대회 발표
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국제학술대회 발표: Hyun Seob Cho, Jin-Hyung Lee, Shi Yong Ryu, Sang Woo Joo, Moo Hwan Cho, Jintae Lee, Antibiofilm activities of *Carex* family and its metabolites against *Pseudomonas aeruginosa* American Society of Microbiology, Boulder CO USA 2013년 5월 19일

국제 논문 발표 1: Hyun Seob Cho, Jin-Hyung Lee, Shi Yong Ryu, Sang Woo Joo, Moo Hwan Cho, Jintae Lee, Inhibition of *Pseudomonas aeruginosa* and *Escherichia coli* O157:H7 biofilm formation by plant metabolite ε-viniferin **Journal of Agricultural and Food Chemistry** 61, 7120-7126 (2013) **(농학 분야 JCR 상위 1.7% 인 세계최고의 저널), 농림수산부 사사**

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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−

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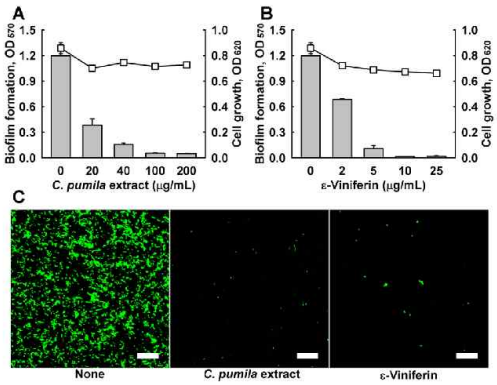

Figure 5. Effects of *C. pumila* extract and *ε*-viniferin on *E. coli* O157:H7 biofilm formation. Biofilm formation (OD_{570}) of *E. coli* O157:H7 was quantified in the presence of *C. pumila* extract (A) and *ε*-viniferin (B) after 24 h in 96-well plates. Cell growths of *E. coli* O157:H7 in the presence 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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Notes

The authors declare no competing financial interest.

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국제 논문 발표 2: Jin-Hyung Lee, Joo-Hyeon Park, Moo Hwan Cho,Jintae Lee, Flavone reduces the production of virulence factors staphyloxanthin and α-hemolysin in *Staphylococcus aureus,* Current Microbiology, 65, 726-732 (2012) **농림수산부 사사**

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Flavone Reduces the Production of Virulence Factors, Staphyloxanthin and α-Hemolysin, in *Staphylococcus aureus*

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Abstract *Staphylococcus aureus* is a leading cause of nosocomial infections due to its resistance to diverse antibiotics. This bacterium produces a large number of extracellular virulence factors that are closely associated with specific diseases. In this study, diverse plant flavonoids were investigated to identify a novel anti-virulence compound against two *S. aureus* strains. Flavone, a backbone compound of flavonoids, at subinhibitory concentration (50 μg/mL), markedly reduced the production of staphyloxanthin and α-hemolysin. This staphyloxanthin reduction rendered the *S. aureus* cells 100 times more vulnerable to hydrogen peroxide in the presence of flavone. In addition, flavone significantly decreased the hemolysis of human red blood by *S. aureus*, and the transcriptional level of α-hemolysin gene *hla* and a global regulator gene *sae* in *S. aureus* cells. This finding supported the usefulness of flavone as a potential antivirulence agent against antibiotic-resistant *S. aureus*.

Introduction

Staphylococcus aureus is an important human pathogen that often exhibits antibiotic resistance and is responsible for worldwide outbreaks of nosocomial infections [14]. This pathogen can secrete several exotoxins, such as hemolysin, enterotoxins, coagulase, TSST-1, and protein A, which are associated with specific diseases [16]. *S. aureus* strains are also capable of producing the golden carotenoid pigment, staphyloxanthin that acts as a virulence factor, primarily being a bacterial antioxidant which protects the pathogen from the host's immune system in the form of reactive oxygen species [3, 13].

Over the past several decades, numerous antibiotics have been developed and used for bacterial infections. However, there has been a significant decrease in the rate of discovery of new antibiotics [13]. Furthermore, current usage of bactericidal compounds is often unsuccessful because of the emergence of methicillin-resistant *S. aureus* [2, 12]. Hence, unlike antibiotics that mostly aim to inhibit cell growth, alternative approaches such as antivirulence compounds have attracted strong research interest. The antivirulence approach aims to reduce the production of virulence factors without affecting bacterial growth to impede the possible emergence of drug resistance [2, 7].

Major discoveries in the antivirulence approach against *S. aureus* include the inhibition of (i) the virulence factor staphyloxanthin [13], (ii) enterotoxins and hemolysins [24] (iii) antibiotic resistant biofilm formation [1, 8, 9], and (iv) bacterial quorum sensing [18]. Recently, several plant compounds have been reported to decrease the virulence of *S. aureus* without affecting its growth. For example, thymol found in thyme [20] reduced enterotoxins and α-hemolysin production; luteolin [19] and chrysin [23] reduced α-hemolysin production; and fisetin [5] and olelic acid [22] inhibited the biofilm formation in *S. aureus*.

The overall aim of this study was to identify novel and potent antivirulence compounds from the screening of plant flavonoids against *S. aureus*. We investigated the effects of 12 flavonoids on the production of virulence factors, such as staphyloxanthin and α-hemolysin in *S. aureus*. Among the tested flavonoids, a subinhibitory concentration of flavone was identified as the most potent

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They are biologically active in combating diseases in humans because of their diverse biological functions, such as antioxidative, antifungal, antiviral, antibacterial, and anti-carcinogenic activities [4]. As the daily dietary intake of mixed flavonoids is estimated to be in the range of 500–1,000 mg [21], they are likely to have minimal toxicity to humans [4], but further study is warranted to confirm this. Recently, the flavonoids luteolin [19] and chrysin [23] at subinhibitory concentrations showed an ability to inhibit the hemolysis of *S. aureus*, and fisetin reduced the antibiotic-resistant biofilm formation in *S. aureus* [5], which demonstrated the potential antivirulence activity of these flavonoids. Compared with luteolin, chrysin, and fisetin, flavone specifically reduced the virulence factor of staphyloxanthin and the H_2O_2 resistance. Therefore, the present results have expanded the scope of previous studies and demonstrated that the functional groups of flavonoids differentially control several virulent phenotypes of *S. aureus*. Flavone is the simplest form among flavonoids used in this study. Although it is speculative, only this simple flavone can be easily transported into *S. aureus* cells and bound to regulatory proteins, while other larger flavonoids may have a transport problem into cells and have a less binding affinity to some regulatory proteins. Further investigation is required to understand how flavone rather than other larger analogs specifically works in *S. aureus* cells.

The expansion in bacterial resistance to antibiotics has created an urgent need for effective antimicrobial agents as well as antivirulence compounds against pathogenic bacteria. In this study, the dual screening of 12 flavonoids for two virulence factors was performed against *S. aureus*, and flavone demonstrated potential as a new potent antivirulence compound. Although the exact action mechanisms of flavone's antivirulence activity remains to be determined, the results suggest that the screening of a larger library of flavonoids will generate more potent therapeutics for the human pathogen *S. aureus*, and possibly for other pathogens as well. Recently, the flavonoid phloretin, which is abundant in apples, reduced the attachment of *Escherichia coli* O157:H7 to human colonic epithelial cells and also diminished colon inflammation in a rat model [11]. Therefore, natural flavonoids are important sources for antivirulence compounds and flavone can be used as a basic structure in the design of potent antivirulence drugs.

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제 5 장　연구개발 성과 및 성과활용 계획

□ 새로운 항병원성 및 항생물막제제의 발굴은 항생제를 대신할 수 있는 신약으로 이용될 수 있으며, 약물내성을 가진 세균에 대해서도 적용 가능하므로 개발 물질의 경제적 가치는 항생제 시장에서 매우 클 것임. 또한 생물막 억제제를 기존의 항생제와 함께 병행하여 항생효과를 극대화할 수 있음.

□ 병원성 세균 관련 질환 예방 및 치료용 물질 분리 동정 기술 축적. 새로운 병원성 억제제의 발견과 그 작용 기전 규명을 통해 안전하고 강력하며 새로운 병원성 저해제 개발 기술을 구축할 것이며, 향후 신약개발에 필요한 효능 평가 기술 축적에 이바지 할 것으로 사료됨.

□ 식물-세균간의 상호작용을 이해할 수 있으며 나아가 동물-세균간 상호작용의 이해를 도움.

□ 항감염제는 인체뿐만 아니라 가축의 사육 (닭, 돼지, 소) 및 양식 (물고기)등에 응용할 수 있어 농림수산분야에 추후 사용 가능함

□ 세균의 독성을 억제하며 패혈증 치료제 개발의 새로운 후보물질을 개발하는 제반연구를 수행함으로써 연구원의 경험과 연구 수행 능력의 향상을 도모하게 되어 21세기 지식 기반 사회의 제일 중요한 구성요소인 경험이 축적된 신약개발 연구 인력의 공급을 통한 산업체 기술력 증대에 일익을 담당함.

□ 선도물질 도출. 선도물질이 도출됨에 따라 세균성 질환의 예방 및 치료제의 개발에 기여하리라 사료됨.

□ 본 연구에서 도출된 새로운 항병원성 및 항생물막제제는 인체 세균감염성 질환을 예방 또는 치료하는데 중요한 정보를 제공할 것임. 항병원성 물질의 발굴은 항생제를 대신할 수 있는 신약으로 이용될 수 있으며, 약물내성을 가진 세균에 대해서도 적용 가능하므로 개발 물질의 경제적 가치는 항생제 시장에서 매우 클 것임.

□ 현재 유럽, 호주, 미국의 연구단체 및 회사에서 병원성 세균의 항병원성물질로 사용하기 위한 상업적 컨소시움이 활발히 진행되고 또한 전 세계적으로 약물내성을 갖는 항생제와는 달리 약물내성 가능성이 적은 새로운 항병원성물질의 탐색이 큰 각광을 받고 있음.

□ 식물추출물의 항생물막 및 병원성 효과에 대한 연구는 식물-미생물간 상호작용에 대한 이해를 도울 것임. 또한 세균의 대장상피세포의 부착 실험은 미생물-인간세포간의 상호작용에 대한 이해를 도와 세균 감염성 질환의 치료 및 예방 후보 물질을 도출할 것임.

□ 세균감염의 최종 문제인 혈액 내 독성 및 적혈구 용혈을 억제하는 물질 개발로서 세균감염 치료를 위한 항생제/병원성 억제물질과의 동시 투여로 세균감염 극복을 극대화할 수 있음.

□ 본 연구는 500종 이상의 다양한 자생식물소재를 이용하여 세 종류의 병원성 미생물의 항병원성물질의 탐색으로 시작하여 다른 병원성 세균에 적용, 확대할 수 있으므로 구축된 식물소재와 한방소재의 활용을 극대화할 수 있는 가능성이 있음.

□ 본 연구의 수행으로 세균의 항병원성 연구 분야에서 세계적인 연구를 선도하고 미생물학과 면역학을 기반으로 새로운 천연물 의약소재 후보물질 탐색에 필요한 경험과 연구 수행 능력을 가진 우수한 생명과학 전문가들을 양성하고자 함.

□ 상용화 계획 및 특허출원을 위한 전략: 본 연구에서 발견한 물질(resveratrol, vinifierin, flavone)은 이미 잘 알려진 물질로써 물질 특허는 어려우며, 해당물질의 효능성과 기능성을 강조하여 특허 출원하고자 함. 또한 해당물질의 출처인 좀보리사초는 현재 생물학적인 특허가 등록돼지 않아 좀보리사초의 병원성억제 효능을 강조하여 특허출원을 완료하고자 함.

제 6 장 연구개발과정에서 수집한 해외과학기술정보

본 과제를 수행하는 동안 지속적으로 다양한 생물막억제 방법에 관한 논문을 접할 수 있었으며 또한 분야별로 본 연구자의 주연구 분야인 의학과 미생물학 분야 뿐만 아니라 생물공학, 환경공학, 재료공학, 식품공학, 해양공학, 생태학, 식물학, 농학 등 다양한 분야의 기술정보를 수집 할 수 있었음. 다양한 참고논문은 아래의 8장 참조.

제 7 장 연구시설·장비 현황

본 연구에서 구입했거나 개발한 연구시설・장비 없음

제 8 장 참고문헌

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