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Antifungal and antibiofilm activities of flavonoids against *Candida albicans*: Focus on 3,2′-dihydroxyflavone as a potential therapeutic agent

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ABSTRACT

Effective management of microbial biofilms holds significance within food and medical environments. *Candida albicans*, an opportunistic fungus, forms mucosal biofilms closely linked to candidiasis and drug-resistant infections due to their drug tolerance. Morphologic change from yeast to filamentous cells is a key virulence factor and a prerequisite for biofilm development. This study investigated the anti-fungal and antibiofilm activities of 20 flavonoids against *C. albicans*. With their known antioxidant capabilities, flavonoids hold promise in combating infections associated with biofilms. Among them, flavone and its derivatives exhibited moderate antifungal activity, 3,2′-dihydroxyflavone (3,2′-DHF) at 1 μg/mL exhibited strong antibiofilm activity (MIC 50 μg/mL). In addition, 3,2′-DHF dramatically inhibited cell aggregation and germ tube/hyphae formation. Transcriptomic analyses revealed that flavone and 3,2′-DHF behaved differently, as 3,2′-DHF downregulated the expressions of germ tube/hyphae-forming and biofilm-related genes (*ECE1*, *HWP1*, *TEC1,* and *UME6*) but upregulated the biofilm/hyphal regulators (*CHK1*, *IFD6*, *UCF1*, and *YWP1*). Tests evaluating toxicity with plant and nematode models revealed that flavone and 3,2′-DHF exhibited mild toxicity. Current results indicate that hydroxylated flavone derivatives can enhance anti-fungal and antibiofilm activities and provide a source of potential anti-fungal agents against drug-resistant *C. albicans*.

1. Introduction

Most microbes can form aggregates of microbial cells encased in a self-produced polymer matrix. These microbial biofilms are ubiquitous in nature and play important roles in the environment and agricultural, food, and medical industries [1–3]. Biofilms may be beneficial in the environment and agricultural contexts but problematic in food and medical settings because they impart drug tolerance and host immune system tolerance. Therefore, novel antibiofilm agents have been actively sought for the last decades.

Candida albicans is an opportunistic pathogen that generally presents on mucosal flora but can also survive in soil and water for around a month [4,5]. *C. albicans* exhibits dimorphism, being able to thrive as either yeast or filamentous cells (germ tube/hyphae), with the latter serving as a pivotal virulence factor essential for biofilm formation [6]. Since drug-resistant *C. albicans* is now widespread, novel therapeutics that target virulence traits and modulate host immunity are being explored to combat anti-fungal resistance [7,8]. *C. albicans* exhibits numerous virulence factors, including adhesins, secreted aspartyl proteases, lipases, phospholipases, as well as the formation of hyphae and biofilms [9]. In particular, the control of *C. albicans* biofilms on host tissues and indwelling medical devices presents a substantial challenge. Diverse approaches have been devised, and the antibiofilm effects of natural small molecules have been extensively reported [10,11].

Flavonoids represent diverse categories of polyphenols present in a multitude of plants, fruits, and vegetables. More than 5000 naturally occurring flavonoid derivatives containing diverse hydroxyl and oxygen groups have been identified [12]. Renowned for their antioxidant prowess, flavonoids are recognized for their ability to scavenge reactive oxygen species (ROS), thereby neutralizing their detrimental impact and shielding cells from oxidative harm. By effectively scavenging ROS, flavonoids contribute significantly to the maintenance of cellular redox balance, thereby safeguarding DNA, proteins, and lipids from oxidative stress-related damage [13–15]. While most plant flavonoids are beneficial dietary components with antioxidant activities and low toxicity, but their low bioavailabilities, caused by poor solubility and absorption coefficients, are limitations. Notably, over 80 flavonoids have been identified for their antifungal effects against a wide spectrum of

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pathogenic fungal strains [12,16]. For example, the MICs of flavone against *Candida* strains were reported to range from 62.5 to 83 μg/mL [17]. Their anti-fungal mechanisms include membrane disruption, inhibition of cell wall synthesis and cell division, mitochondrial dysfunction, and the inhibition of RNA/DNA and protein synthesis [12]. Recently, a number of studies have investigated the antibiofilm activities of flavonoids against *C. albicans*. For example, prenylated flavonoids and flavanones [18–20], baicalein and quercetin [21,22], polyphenols [23,24], and flavonoid-rich fractions of *Bauhinia holophylla* leaves [25] inhibited biofilm formation by *C. albicans*. Hence, the present study was designed to investigate the antibiofilm effect of 20 flavonoids, including flavonols, flavones, flavonones, and isoflavonoids, against two *C. albicans* strains, and to further investigate the responsible mechanisms.

After initial screening, the most active 3,2-dihydroxyflavone (3,2′-DHF) found from *Marsdenia tinctoria* R. Br. (Apocynaceae) was selected, and its activity was then compared with flavone. Live imaging microscopy and scanning electron microscopy (SEM) were employed to observe the inhibition of biofilm formation and alterations in cell morphology. Additionally, qRT-PCR was utilized to explore the molecular mechanisms underlying their antibiofilm activities. Chemical toxicities were investigated using plant and nematode models.

2. Materials and methods

2.1. Candida strains

DAY185 (fluconazole-resistant) and ATCC 10231 (fluconazole-sensitive) strains of *C. albicans* were sourced from the Korean Culture Centre for Microorganisms (KCCM) and the American Type Culture Collection (ATCC), respectively. Cultivation was carried on solid potato dextrose agar (PDA) for colony production through streaking from glycerol stock (glycerol 15 %) at −80 °C. For all other experiments, liquid potato dextrose broth (PDB) was utilized. In addition, YPD, Saboraud, and RPMI media were used for antibiofilm activities of two hits. After incubating cells streaked on PDA plates for 2 days at 37 °C, a single colony was chosen. This colony was then inoculated into 25 mL of PDB in 250 mL Erlenmeyer flasks, undergoing 48 h culture at 37 °C.

2.2. Reagents

The investigation encompassed 20 flavonoids, listed as follows; 6-aminoflavone, catechin, curcumin, 2,3-dihydroflavone, 3,2′-dihydroxyflavone (3,2′-DHF), 5,7-dihydroxyflavone, 7,2′-dihydroxyflavone, 4′,7-dihydroxyisoflavone, 2,2′-dihydroxy-4-methoxybenzophenone, 2,2′-dihydroxy-4,4′-dimethoxybenzophenone, epicatechin, flavone, 6-hydroxyflavone, 7-hydroxyflavone, naringin, phloretin, quercetin, 3,3′,4′,7-tetrahydroxyflavone, 4′,5,7-trihydroxyflavone, and 4′,5,7-trihydroxyisoflavone. Flavonoids were purchased from Sigma-Aldrich (St. Louis, USA), Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan), or Combi-blocks (San Diego, USA) and dissolved in dimethyl sulfoxide (DMSO). The DMSO levels in cell cultures were kept below 0.1 % (v/v), which did not affect cell growth or biofilm formation of *C. albicans*.

2.3. MICs

To assess planktonic cell growth, measurements were taken using a spectrophotometer (Multiskan SkyHigh microplate reader; Thermo Fisher Scientific, Waltham, MA, USA) at 620 nm following a 24 h cultivation at 37 °C. A slightly modified version of the Clinical Laboratory Standards Institute (CLSI) broth dilution method was used to determine minimum inhibitory concentrations (MICs) [26,27]. Briefly, overnight cultivation of *Candida* strains was conducted in 250 mL flat-bottomed flasks containing 25 mL of PDB medium. Cells were then diluted 1:100 in a 0.5 McFarland suspension and further diluted at 1:20 in RPMI-1640 (Thermo Fisher Scientific Inc., Waltham, MA, USA). Mixtures were then dispensed into wells of a 96-well polystyrene plate (polystyrene) containing different concentrations (w/v) of the flavonoids and incubated for 24 h at 37 °C. MIC was defined as the lowest concentration in which planktonic cell growth was undetectable. Four independent experiments with six replicates were performed at each concentration.

To assess the fungicidal effect of 3,2′-DHF on *C. albicans*, overnight cultures were reinoculated at a 1:50 dilution with or without 3,2′-DHF at concentration of 1xMIC (50 μg/mL) and 5xMIC (250 μg/mL) and incubated at 37 °C. The cultures were then serially diluted using appropriate dilution factors in PBS and plated on PDA plates at 0, 2, 4, 8, and 24 h intervals. Colony-forming units (CFU) on the plates were counted after incubation and CFU per mL was calculated using the formula: CFU/mL = Number of colony units × dilution factor/volume plated (mL).

2.4. Biofilm inhibition and eradication assays

C. albicans biofilms were developed on 96-well plates, as previously reported [28]. Cultures of *C. albicans* cells, incubated overnight, were sub-inoculated into fresh PDB (300 μL) at an optical density of 0.1 (~10^6 CFU/mL) at 600 nm in 96-well plates. The cells were then incubated with or without 10 and 50 μg/mL concentrations of the 20 flavonoids for 24 h without agitation at 37 °C. Afterward, planktonic cells and spent medium were discarded, and the plates were washed three times with distilled water to eliminate free-floating cells. Biofilm cells on the well surfaces were subsequently stained with crystal violet (0.1 % w/v, Sigma-Aldrich, St. Louis, USA) for 20 min and washed three times with distilled water. The crystal violet was dissolved using 95 % ethanol, and the absorbances of crystal violet in ethanol were measured at 570 nm using a spectrophotometer. Two independent experiments with six replicates were performed at each concentration.

For the biofilm eradication/dispersal assay, established biofilms of *C. albicans* DAY185 were formed over 4 h. Flavonoids were then introduced at concentrations up to 8 times of MIC, and after an additional 24 h of incubation, biofilm formation was measured as described above. Two independent experiments with six replicates were performed at each concentration.

Combinatorial efficacies of 3,2′-DHF and antifungal agents were analyzed. Briefly, overnight cultures of *C. albicans* at an initial turbidity of OD 0.1 at 600 nm were inoculated into a PDB with antifungal agents, amphotericin B (0.05, 0.1, 0.2, 0.5, and 1 μg/mL) or butaconazole (0.5, 1, 2, 5, and 10 μg/mL) and/or 3,2′-DHF (0.5 and 100 μg/mL). Cells were incubated for 24 h at 37 °C. Biofilm formation was determined by crystal violet staining like above. Two independent experiments with six replicates were performed at each concentration.

2.5. Cell viability assay - XTT reduction assay

Cell viability in biofilms were analyzed using a 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide sodium salt (XTT) according to manufacturer's method (Cell proliferation kit, Sigma-Aldrich). Biofilms were established as described above, by culturing the cells for 24 h at 37 °C without shaking with or without flavones. The metabolic activities were measured by adding 100 μL of freshly prepared mixture of XTT labeling reagent and electron-coupling reagent (phenazine methosulfate) at 50:1 (v/v) after rinsing each well with sterile water for removing non-biofilm cells and spent medium. Biofilm cells in 96-well plate were incubated at 37 °C in the dark for 1 h. Colorimetric change was measured by a Multiskan EX microplate reader at 490 nm. Planktonic cell growth was measured at 600 nm before starting XTT assay. Results were averaged from at least three independent cultures. Two independent experiments with six replicates were performed at each concentration.

2.6. Microscopic observations of C. albicans biofilm formation

C. albicans DAY185 biofilms were cultivated for 24 h at 37 °C, following the previously outlined procedure. Subsequently, free-floating cells and spent medium were gently eliminated through three rounds of washing with distilled water. Biofilms cells were moisturized with 50 μL of PBS and then visualized by live cell imaging microscopy using the iRiS™ Digital Cell Imaging System (Logos BioSystems), and color-coded 2D and 3D images were generated using ImageJ (https://imagej.nih.gov/ij) [26]. Four independent experiments with six replicates were performed at each concentration.

2.7. C. albicans germ tube/hyphae formation and cell aggregation

C. albicans DAY185 cells were sub-inoculated into 0.5 mL of PDB at a cell density of 10^6 CFU/mL in 1.7-mL microcentrifuge tubes with or without flavonoids and incubated without agitation for 24 h at 37 °C [29]. The plate spreading which involves distributing bacteria evenly over the surface of an agar plate was used for enumeration. Cell cultures (0.5 mL) were gently mixed by tapping, transferred to slide glass, and observed in bright field under an optical microscope, iRiS™ Digital Cell Imaging System (Logos BioSystems, Anyang, Republic of Korea). At least four independent experiments were conducted.

SEM was used to analyze hyphae development, as previously reported [28]. A sterile nylon membrane (0.22 μm pore size, Whatman, Maidstone, UK) was cut into small pieces (~0.4 × 0.4 cm), and then single pieces were placed on the bottom of wells of 96-well plates containing 300 μl of cell culture of turbidity 0.1 at 600 nm. Cells were then incubated with or without each flavonoid for 24 h at 37 °C without shaking. Biofilm cells formed on nylon membrane pieces were fixed with a mixture of glutaraldehyde (2.5 %) and formaldehyde (2 %) for 20 h at 4 °C and then dehydrated using an ethanol series (50, 70, 80, 90, 95, and 100 %) for 40 min per step. After critical-point drying (EM CPD300 Critical point dryer, Leica Biosystems, Wetzlar, Germany) and coating (E1030-Ion sputter coater, Hitachi, Japan), biofilm cells on nylon membranes were visualized under an FE-SEM (field emission scanning electron microscope) (S-4800, Hitachi) at a voltage of 15 kV and magnifications of 500 to 5000x. Two independent experiments with six replicates were performed at each concentration.

2.8. RNA isolation and quantitative real-time PCR (qRT-PCR)

For transcriptomic analysis, *C. albicans* DAY185 was introduced into 9 mL of fresh PDB within 250 mL Erlenmeyer flasks at an OD_{600} of approximately 0.1. The culture was then incubated for 6 h at 37 °C with shaking at 250 rpm, either in the presence or absence of 3,2′-DHF or flavone (10 μg/mL). To prevent RNA degradation, RNase inhibitor (RNAlater®; Ambion, TX, USA) was gently added and mixed before cell harvesting. Total RNA was isolated from *C. albicans* using acidic phenol (pH 4.5–5.5) at 65 °C, purified using an RNeasy mini Kit (Qiagen, Hilden, Germany), as previously described [28], and analyzed using a NanoVue UV–Vis spectrophotometer (GE Healthcare, Chicago, USA) for concentration and purity. qRT-PCR was conducted with gene-specific primers to assess the expression levels of genes associated with hyphae and biofilm formation. (*ALS1, ALS3, ECE1, EFG1, HWP1* (also called *ECE2*), *IFD6, RBT1, TEC1, UCF1, UME6, YWP1,* and *ZAP1*). *RDN18* served as the internal control for normalization purposes. (Table S2). qRT-PCR was conducted using M-MLV reverse transcriptase (Thermo Fisher Scientific, Waltham, USA), RNase inhibitor (Thermo Fisher Scientific), SYBR Green real-time PCR master mix (Thermo Fisher Scientific), and a StepOne Real-Time PCR System (Applied Biosystems, Foster City, USA) [28]. Two independent experiments with four replicates were performed at each concentration.

2.9. Chemical toxicity assays using the seed germination model

The germination and growth of Chinese cabbage (*Brassica rapa* L.) were used to explore the toxicities of flavonoids, as previously reported [26]. In summary, *B. rapa* seeds underwent a three-time wash with sterile water, followed by a 20 h soak in sterile water. Subsequently, the seeds were surface sterilized through sequential incubation in 95 % ethanol and 3 % sodium hypochlorite for 20 min at room temperature and underwent five washes with sterile water. These treated seeds were then placed on soft agar Murashige and Skoog plates (0.7 % agar and 0.86 g/L Murashige and Skoog) with or without flavonoids, and the plates were incubated at room temperature (23 °C) for a duration of 5 days. Seed germination rates and total plant lengths were measured on a daily basis. Four independent experiments were performed at each concentration.

2.10. Chemical toxicity assays using the nematode model

To investigate the chemical toxicities of flavonoids, *C. elegans* strain *fer-15(b26)*; *fem-1(hc17)* was used, as previously described [29]. Briefly, synchronized young adult nematodes were cleaned twice with M9 buffer (3 g/L KH_2PO_4, 6 g/L Na_2HPO_4, 5 g/L NaCl, 1 mM $MgSO_4$), and ~30 worms were dispensed into each well of 96-well plates containing M9 buffer (200 μL) and flavonoids (20 or 100 μg/mL). Plates were then incubated for 10 days at 25 °C without agitation under dark condition. Four independent experiments were performed in triplicate. Results are presented as percentages of live worms, as decided by responses to plate tapping and LED lights for 20–30 s using the iRiS™ Digital Cell Imaging System (Logos BioSystems). Two independent experiments with six replicates were performed at each concentration.

2.11. Statistical analysis

Statistical analysis was conducted using one-way analysis of variance (ANOVA) followed by Dunnett's test, implemented in SPSS version 23 (SPSS Inc., Chicago, IL, United States). Significance was determined at P values < 0.05. Asterisks denote significant differences between untreated and treated samples, and results are expressed as means ± standard deviations. The number of sample replicates is indicated above.

3. Results

3.1. Antibiofilm activities of the 20 flavonoids against C. albicans

The inhibitory effects of the 20 flavonoid derivatives at 10 or 50 μg/mL on biofilm formation by fluconazole-resistant *C. albicans* DAY185 strain were initially determined using 96-well polystyrene plates. Of the 20 flavonoids, 6-aminoflavone, 2,3-dihydroflavone, 3,2′-DHF, 2,2′-dihydroxy-4-methoxybenzophenone, and flavone at 50 μg/mL inhibited *C. albicans* biofilm formation more than 85 % (Fig. 1A and B). In particular, 3,2′-DHF at 10 μg/mL completely inhibited *C. albicans* biofilm formation, and flavone at 10 μg/mL also inhibited biofilm formation by > 84 %. Detailed information on the effects of flavonoids on biofilm formation, cell growth, and MIC against *C. albicans* is provided in Table S1. Notably, 3,2′-DHF, 2,2′-dihydroxy-4-methoxybenzophenone, and flavone at 10 μg/mL had little effect on planktonic cell growth but significantly inhibited biofilm formation. Minimum inhibitory concentrations (MICs) of the 20 flavonoids were measured and are presented in Table S1. The MICs of 3,2′-DHF and 2,2′-dihydroxy-4-methoxybenzophenone were 50 and 100 μg/mL, respectively. Also, it appears that 3,2′-DHF was fungicidal with > $5\log_{10}$ reduction of CFUs after treatment of 3,2′-DHF at 50 μg/mL for 24 h (Fig. S1). The MICs of other flavonoids were >100 μg/mL, but this was difficult to measure due to the poor water solubility of flavonoids. These results suggest that flavonoids with antibiofilm activity, unlike conventional anti-fungal agents, are less prone to the development of drug resistance since they

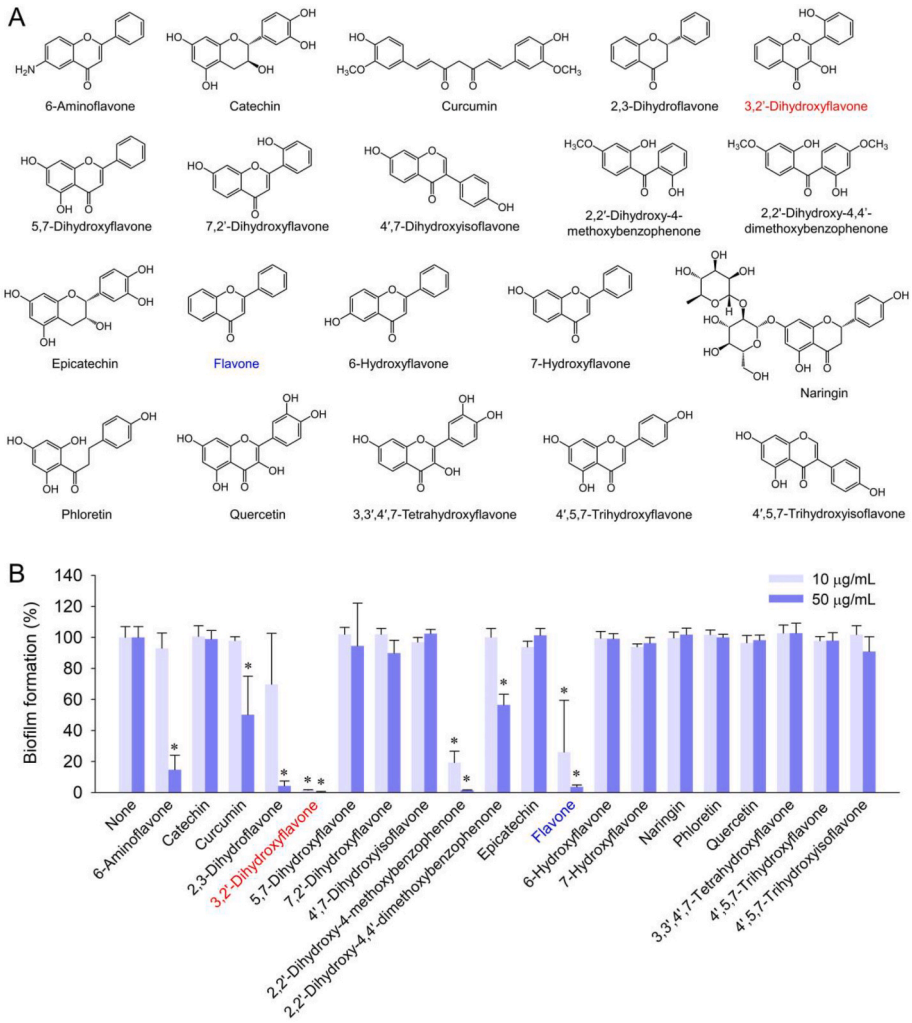

Fig. 1. Antibiofilm activity of flavonoids. Structures of the flavonoids studied (A). Biofilm formation by *C. albicans* DAY185 in the presence of flavonoids at 10 or 50 μg/mL (B). Error bars indicate standard deviations. *, $P < 0.05$ *vs*. nontreated controls (None).

do not target planktonic cell growth and that their antibiofilm activities are due to mechanisms other than their anti-fungal activities. Hence, the most active 3,2′-DHF was selected for further mechanistic studies; flavone was included for comparison purposes.

3.2. Biofilm inhibition by 3,2′-DHF and flavone

A more detailed biofilm study showed that 3,2′-DHF and flavone dose-dependently inhibited biofilm formation by *C. albicans* DAY185 (Fig. 2A and B). For example, 3,2′-DHF at 1 μg/mL inhibited biofilm formation by 75 %, while flavone at ten times this concentration only inhibited biofilm formation by the same amount. Since the results obtained with different media are often different, other three media were used. Results using YPD and Saboraud medium are similar to results with PDB medium as 3,2′-DHF was much active than flavone. However, in RPMI medium, 3,2′-DHF up to 10 μg/mL did not show its antibiofilm activity while flavone showed some activity (Fig. S2).

XTT reduction assays showed that the metabolic activity of biofilm cells was markedly decreased by 3,2′-DHF from 0.5 μg/mL and by flavone from 20 μg/mL (Fig. 2C and D), and these results were in line with biofilm inhibitions. In addition, biofilm inhibition was investigated by live imaging microscopy (Fig. 2E). When untreated, *C. albicans* formed dense biofilms, but in the presence of 3,2′-DHF at > 2 μg/mL or flavone at > 20 μg/mL biofilm formation almost abolished. However, neither 3,2′-DHF nor flavone at concentrations up to 400 μg/mL eradicated established biofilms (Fig. S3), confirming that biofilm eradication is considerably more difficult than inhibiting biofilm formation.

The antibiofilm activities of the 3,2′-DHF or flavone were also tested against a fluconazole-sensitive *C. albicans* ATCC 10231 strain, 3,2′-DHF and flavone also dose-dependently inhibited biofilm formation by the fluconazole-sensitive *C. albicans* ATCC 10231 strain (Fig. S4).

Also, combinatory treatment with antifungal drugs (amphotericin B and butaconazole) has been performed for antibiofilm and biofilm dispersal activities. 3,2′-DHF displayed additive effect rather than synergistic effect for its antibiofilm activity with amphotericin B and butaconazole. However, even combinatory treatment could not lead biofilm dispersal (Fig. S3).

3.3. Inhibitory effects of 3,2′-DHF and flavone on hyphal formation and cell aggregation

Since the development of filamentous hyphae from yeast cells is a crucial virulence factor and a prerequisite for *C. albicans* biofilm formation [30], hyphae transition was investigated in the absence and presence of 3,2′-DHF or flavone. In untreated samples, yeast, pseudohyphae, and hyphal cells were observed in PDB medium (Fig. 3A), and 3,2′-DHF at > 2 μg/mL or flavone at > 20 μg/mL completely inhibited hyphae formation. Also, cell aggregation was measured in PDB. In line with hyphae results, nontreated controls formed massive cell aggregates and 3,2′-DHF or flavone dose-dependently inhibited this aggregation

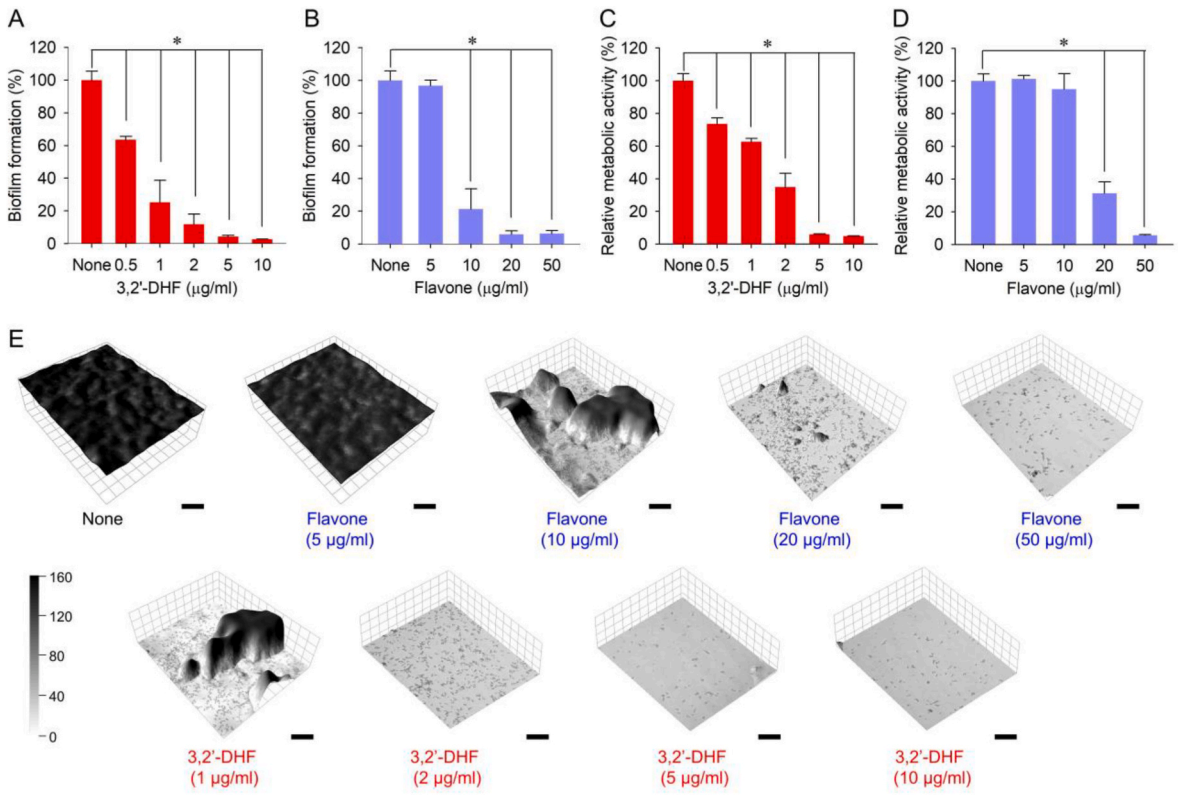

Fig. 2. Antibiofilm activities of 3,2′-dihydroxyflavone (3,2′-DHF) and flavone. Antibiofilm activities of 3,2′-DHF (A) and flavone (B) and effects of 3,2′-DHF (C) and flavone (D) on the metabolic activity of *C. albicans* DAY185 via XTT reduction assay. 3-Dimensional biofilm images of *C. albicans* DAY185 in the presence of 3,2′-DHF or flavone (E). Black scale bar represents 100 μm *, $P < 0.05$ *vs.* nontreated controls (None).

(Fig. 3B). Further SEM analysis confirmed that 3,2′-DHF or flavone inhibited hyphae transition and cell aggregation (Fig. 4). These findings strongly corroborate the idea that 3,2′-DHF or flavone hinders hyphal transition and cell aggregation, consequently impeding the formation of biofilms by *C. albicans*.

3.4. Effects of 3,2′-DHF and flavone on the expressions of biofilm-related genes

qRT-PCR was performed to investigate the molecular bases of the inhibitions of biofilm formation and hyphal transition by 3,2′-DHF or flavone. The expressions of 13 biofilm- and hyphae-related genes were quantified after treating *C. albicans* DAY185 with 3,2′-DHF or flavone at 10 μg/mL for 6 h. *RDN18* (18S ribosomal RNA) was used as the housekeeping gene. Interestingly, the patterns of expressional changes induced by 3,2′-DHF and flavone differed as flavone downregulated several genes while 3,2′-DHF downregulated or upregulated biofilm- and hyphae-related genes (Fig. 5A). Specifically, 3,2′-DHF significantly suppressed the expression of *ECE1* (hyphae-specific protein, also called *HWP2*) by 28-fold, *HWP1* (hyphal cell wall protein, also called *ECE2*) by 18-fold, and *UME6* (filament-specific regulator) by 6.6-fold, whereas 3,2′-DHF increased the expressions of *CHK1* (*C. albicans* histidine kinase 1) by 4.6-fold, *IFD6* (an inhibitor of biofilm matrix production) by 15-fold, *UCF1* (upregulated by cAMP in filamentous growth) by 9.4-fold, and *YWP1* (yeast form wall protein 1) by 7-fold. In contrast, flavone significantly suppressed the expression of *ALS1* (agglutin-like protein 1) by 5.6-fold, *ALS3* (agglutin-like protein 3) by 13-fold, *ECE1* by 18-fold, *HWP1* by 4.5-fold, *IFD6* by 10-fold, and *UME6* by 6.1-fold. These results demonstrate 3,2′-DHF and flavone at sub-inhibitory concentrations markedly influenced the gene expressions associated with biofilm and hyphal formation and thus inhibited *C. albicans* biofilm and hyphae development.

3.5. Effects of 3,2′-DHF and flavone in the plant and nematode models

A plant growth model and a nematode survival model were used to study the environmental effects and chemical toxicities of 3,2′-DHF and flavone. The seed germination rate of *B. rapa* was not affected by 3,2′-DHF or flavone at concentrations up to 100 μg/mL (Fig. 6A), while plant lengths were slightly reduced by 3,2′-DHF and flavone (Fig. 6B and C). Mild plant toxicities were expected as 3,2′-DHF and flavone are found in plants such as *Marsdenia tinctoria* [31].

The toxicities of 3,2′-DHF and flavone were also investigated using nematode *C. elegans*. Interestingly, 3,2′-DHF was much less toxic to *C. elegans* than flavone (Fig. 6D). Specifically, most nematodes survived treatment with 3,2′-DHF at 20 μg/mL for 8 days, whereas the majority died after treatment with flavone at 20 or 100 μg/mL. These results indicate that 3,2′-DHF, in its active antibiofilm range (2–10 μg/mL), has only marginal effects on plant growth and nematode survival.

4. Discussion

The present study reports the anti-hyphal and anti-biofilm effects of various flavonoids on *C. albicans* and partially reveals the mechanisms of the most active dihydroxyflavone and flavone. While the anti-fungal activities of various flavonoids have been extensively reported [12,17], the current study reports for the first time that several flavonoids at sub-inhibitory concentrations exert antibiofilm activities against *C. albicans* (Fig. 1 and Table S1). Notably, the anti-fungal activities of flavonoids were moderate, while their antibiofilm activities were potent; for example, 3,2′-DHF at 1 μg/mL significantly inhibited biofilm formation (Fig. 2) without killing *C. albicans* cells (MIC 50 μg/mL). Our results are comparable to previous reports on the antibiofilm activities of other flavonoids, such as prenylated flavonoids [18,19], baicalein, and quercetin [21,22], which at near their MICs (8–64 μg/mL) had

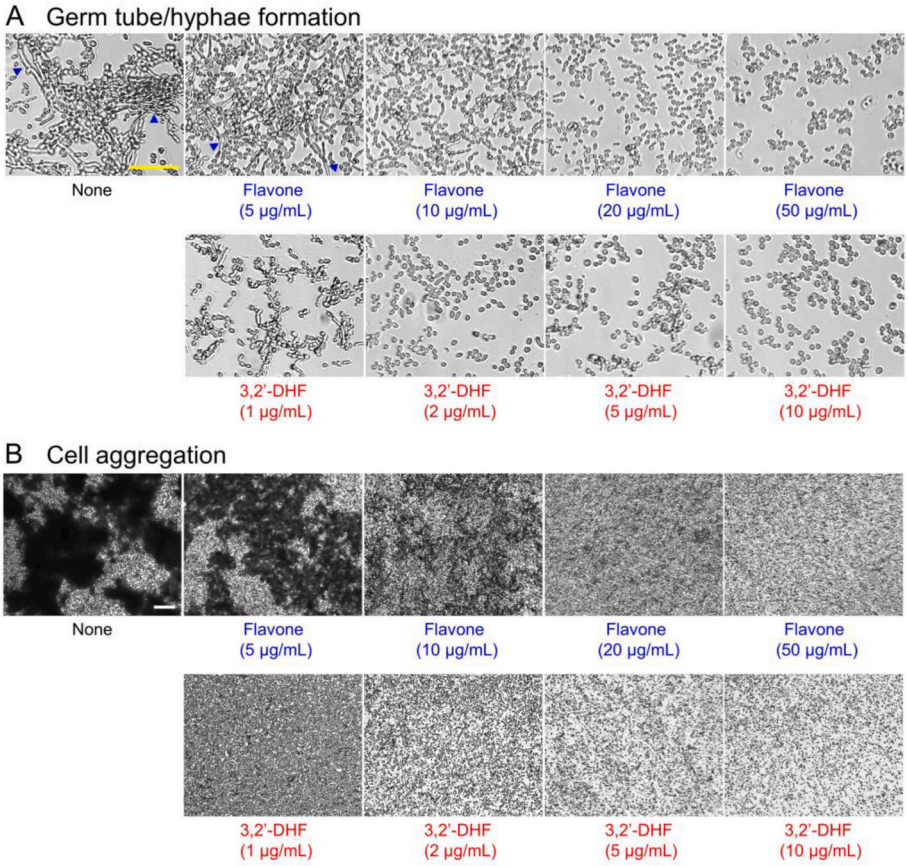

Fig. 3. Inhibitory effects of 3,2′-dihydroxyflavone (3,2′-DHF) and flavone on germ tube/hyphal development and cell aggregation by *C. albicans* DAY185. Germ tube/hyphae formation (A) and cell aggregation (B) using live cell imaging microscopy. Images were captured at 10 to 40× magnifications. Yellow and white bars represent 50 and 200 μm, respectively. Blue triangles indicate germ tube/hyphae formation. Nontreated controls (None).

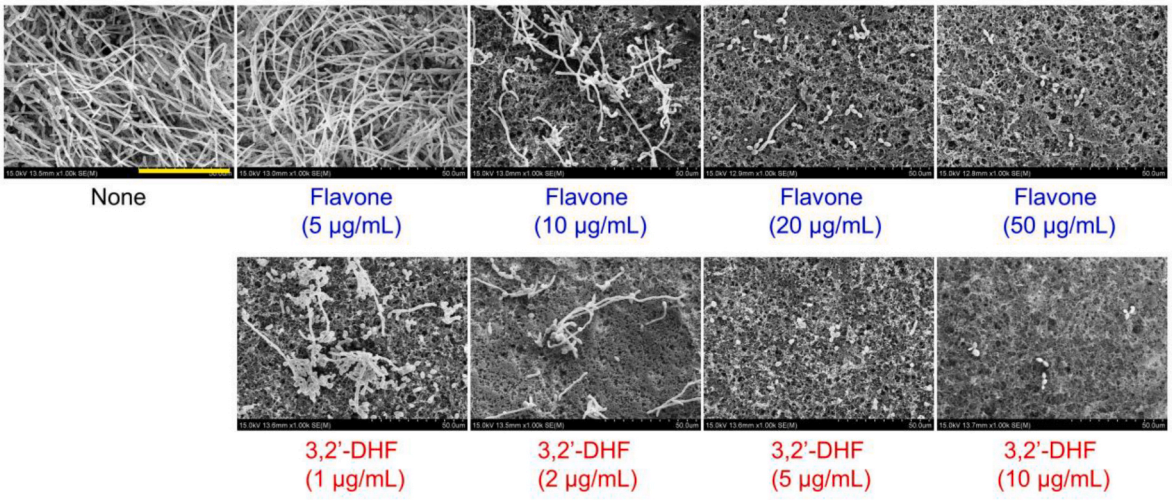

Fig. 4. Inhibitory effects of 3,2′-dihydroxyflavone (3,2′-DHF) and flavone on hyphal growth and biofilm formation by *C. albicans* DAY185 as determined by SEM. The yellow scale bar represents 50 μm. Nontreated controls (None).

antibiofilm activities attributable in part to their anti-fungal activities. More recently, we reported that several flavonoids, including 3,2′-DHF, 2,2′-dihydroxy 4-methoxybenzophenone, 6-aminoflavone, polymethoxyflavones and flavone at concentrations of 10–50 μg/mL exhibited antibiofilm activities against *Vibrio* species and *Staphylococcus aureus* [32,33]. However, their action mechanisms differed from those observed in the present study as they suppressed cell motilities, hydrophobicity, cell aggregation, iron acquisition, and hemolysin production in *Vibrio* species [32]. Therefore, we propose exploring the antibiofilm effects of flavonoids on various microbes and mixed microbial populations.

Of the 20 flavonoids studied, 3,2′-DHF, 2,2′-dihydroxy-4-methoxybenzophenone, flavone, 2,3-dihydroflavone, and 6-aminoflavone (in decreasing order of potency) at 10 or 50 μg/mL showed antibiofilm

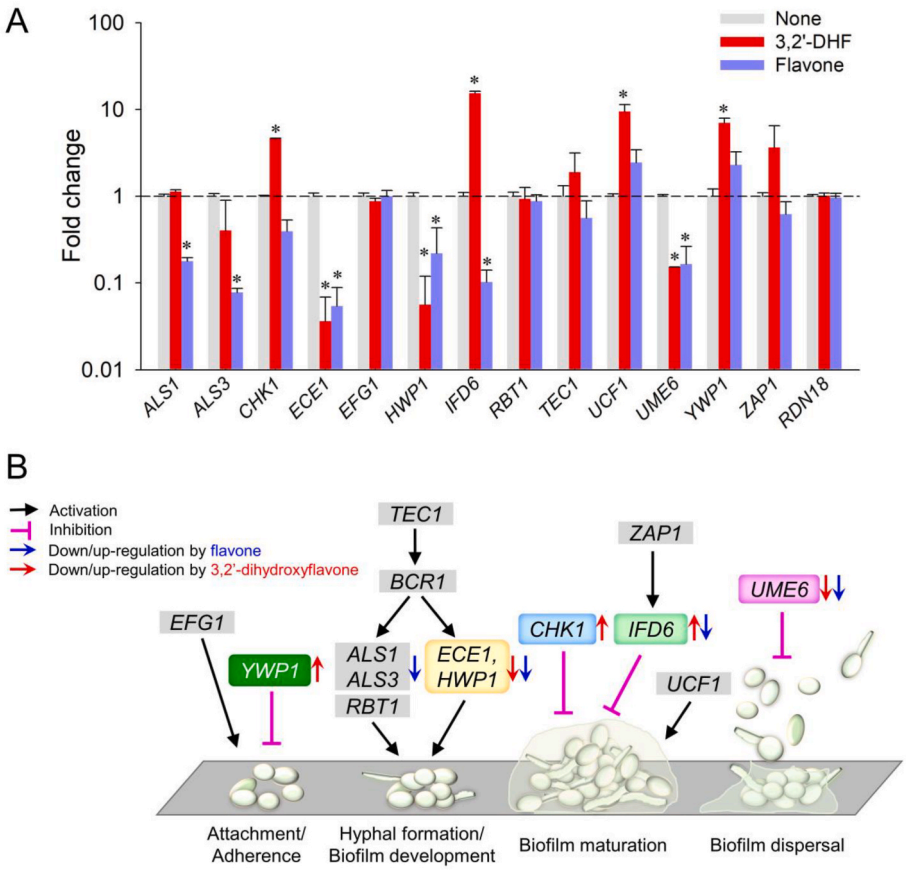

Fig. 5. Differential gene expressions and mechanisms responsible for the effects of 3,2′-dihydroxyflavone (3,2′-DHF) and flavone on *C. albicans* DAY185. Fold changes represent the transcriptional variances identified in cells subjected to treatment compared to those untreated (None), as determined through qRT-PCR. *C. albicans* cells were treated with 3,2′-DHF or flavone at 10 μg/mL for 6 h without shaking (A). *RDN18* was used as the housekeeping gene. *, $p < 0.05$ *vs.* nontreated controls. Potential mechanism underlying the effects of 3,2′-DHF and flavone in *C. albicans* (B).

activity against *C. albicans*, whereas 6-hydroxyflavone, 7-hydroxyflavone, 7,2-dihydroxyflavone, 5,7-dihydroxyflavone (chrysin), and 4′,5,7-trihydroxyflavone (apigenin) did not. It appears that the presence of a hydroxyl group at C3 of the C-ring or at C2' of the B-ring of flavone favors antibiofilm activity and that hydroxylation at C5, C6, or C7 reduces antibiofilm activity. Intriguingly, the introduction of an amino group at C6 also enhanced antibiofilm activity. Since flavones are easily hydroxylated, methylated, isoprenylated, and halogenated [34], we suggest that the activities and bioavailabilities of flavones possessing amino group(s), methyl group(s), or halogen-containing groups at different C positions should be further investigated.

3,2′-DHF and flavone inhibited cell aggregation, hyphae development, and biofilm formation by *C. albicans* (Figs. 2–4), and qRT-PCR results were in line with these phenotypic changes; for example, 3,2′-DHF and flavone suppressed the expressions of several hyphae-related genes, such as *ECE1* (hyphae-specific protein), *HWP1* (hyphal cell wall protein), and *UME6* (filament-specific regulator) (Fig. 5). Furthermore, 3,2′-DHF more dramatically modulated the expressions of biofilm-related genes than flavone.

The mechanism responsible for the inhibitory effect of flavone was found to involve the down-regulations of agglutin-like protein genes (*ALS1* and *ALS3),* hyphal cell wall protein genes (*HWP1* and *ECE1*), a gene related to the inhibition of biofilm matrix production *(IFD6*), and filament-specific regulator (*UME6*), which would result in the inhibition of cell aggregation, hyphal initiation, and biofilm development (Fig. 5). In contrast, 3,2'-DHF upregulated the expression of several genes, such as *CHK1* (*C. albicans* histidine kinase 1), *IFD6* (an inhibitor of biofilm matrix production), *UCF1* (upregulated by cAMP during filamentous growth), and *YWP1* (yeast form wall protein 1) (Fig. 5), which support biofilm inhibition by 3,2'-DHF. The mechanism of hyphae and biofilm development is complex, as shown in Fig. 5B (adopted from studies [26,29,35]). For example, the upregulation of *CHK1* and *IFD6* would suppress biofilm maturation, and the induction of *YWP1* would reduce cell adhesion. *UCF1* is positively regulated by cAMP during filamentous growth [36] and was upregulated by 3,2′-DHF in the present study. Similar effects were induced by medium-chain fatty acids, 6-bromochromone-3-carbonitrile, and nepodin, which also inhibited the biofilm activity of *C. albicans* [29,37,38]. Additional transcriptomic and molecular studies are required to elucidate the exact mechanism involved.

Flavones constitute the largest flavonoid subgroup, are found in various plants, and have diverse effects, which include antioxidative and anticancer activities [34]. 3,2′-DHF is a rare dihydroxyflavone found in a tropical plant *Marsdenia tinctorial* [31] and comparatively little is known about its microbiological activities, although [39,40] reported it has mild antibacterial and anti-fungal activity. Here, we report for the first time that 3,2′-DHF has potent antibiofilm activity against *C. albicans* at concentrations of only a few micrograms per milliliter. In addition to the previously reported beneficial effects of 3,2′-DHF on cell proliferation [41–43], wound healing effects, and skin regeneration [44], and its antiviral effects on influenza virus [45], the present study suggests it might also be useful for treating *Candida*-associated lesions.

Furthermore, our results showed that 3,2′-DHF was not phytotoxic or nematotoxic (Fig. 6A–D). Although further toxicological and clinical studies are required to confirm its anti-fungal effects, the current study suggests 3,2′-DHF might be a potential antifungal, antibiofilm, and antivirulence agent against drug-resistant *Candida* infections.

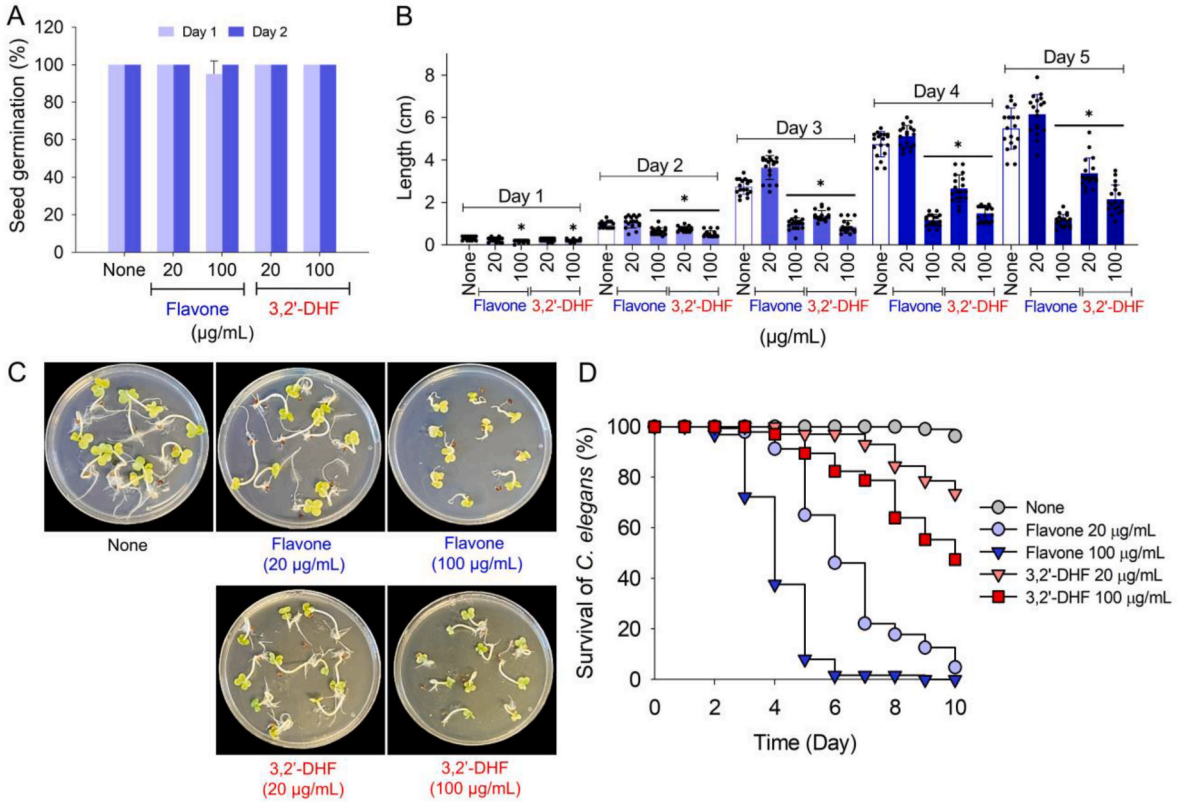

Fig. 6. Chemical toxicities of 3,2′-dihydroxyflavone (3,2′-DHF) and flavone. *B. rapa* seed germination assay was performed with or without 3,2′-DHF or flavone at 25 °C for 2 days (A). Plant total lengths were measured on days 1, 2, 3, 4, and 5 (B and C), and *C. elegans* survival was assessed in the presence or absence of 3,2′-DHF or flavone for 10 days (D). *, $p < 0.05$ *vs.* nontreated controls. Nontreated controls (None).

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CRediT authorship contribution statement

Jin-Hyung Lee: Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Yong-Guy Kim:** Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Resources, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Inji Park:** Software, Formal analysis. **Jintae Lee:** Writing – review & editing, Writing – original draft, Validation, Supervision, Resources, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biofilm.2024.100218.

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