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Autoinducers modulation as a potential anti-virulence target of bacteria by phenolic compounds

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ABSTRACT

Objective: The goal of this study was to determine the effects of phenolic extracts from grape (GrPE), pomegranate (PoPE), and persimmon (PePE) by-products on bacterial virulence activities such as biofilms, motility, energy-dependent efflux pumps, and β-lactamase activity, which are modulated primarily by quorum sensing (QS), defining their potential applications.

Method: The microdilution method was used to determine the minimum inhibitory concentration (MIC) and sub-inhibitory concentrations (SICs) of the extracts against reference pathogenic bacteria. The antibacterial mode of action was determined by labelling bacterial cells in *in vivo* cell-tracking experiments.

Results: Antibiograms showed that PoPE inhibited bacteria at lower concentrations, and PePE had a stronger effect against *Klebsiella pneumoniae*. Both extracts caused significant cell membrane damage (CMD), whereas GrPE did not. At SICs, all extracts showed anti-QS activity, especially PePE, which inhibited violacein and pyocyanin production at 1/128 × MIC. Additionally, QS autoinducers found in *Chromobacterium violaceum* and *Pseudomonas aeruginosa* were modulated by the extracts; PePE showed the highest modulation. Antibiofilm assays revealed that GrPE, at MIC and 2 × MIC, acted as a potent antibiofilm agent against biofilms of *Pseudomonas putida, Bacillus cereus*, and *Staphylococcus aureus*, which was related to disruption of swarming motility by GrPE. All extracts, especially PoPE, exerted a potent effect against the activation of efflux pumps of *P. aeruginosa* as well as β-lactamase activity in *K. pneumoniae*.

Conclusion: Results suggest that the anti-virulence potential of the extracts may be related to their effect as extracellular autoinducer modulators. This study allowed to define potential applications of these extracts.

1. Introduction

Industrial food by-products are a rich source of biocompounds such as polyphenols and carotenoids, among others. These compounds tend to be covalently linked to polysaccharides or free in the fruit matrix; hence, they are usually undervalued and discarded [1]. Grape (*Vitis vinifera* L.), pomegranate (*Punica granatum* L.), and persimmon (*Diospyros kaki* Thunb.) are crops with high economic value. By-products are generated by the processing of these fruits for juice [2], canned food, and other products [3]. The opportunity to upcycle food by-products and turn them into new products benefits the pharmaceutical, biotechnological, and food industries by promoting a circular economy, in line with the United Nations 2030 Agenda for Sustainable Development.

Phenolic compounds are well known for their ability to cause cell membrane damage (CMD) and disrupt enzyme metabolism [4]. However, these compounds can also target additional virulence activities, indicating unexplored and potential applications for phenolic compounds beyond their antibacterial effects [5]. This opens up possibilities for discovering novel anti-virulence targets, which

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is crucial in addressing the current lack of effective antibiotic research and the rapid emergence of bacterial resistance [6].

A promising strategy for disrupting bacterial virulence and attenuating pathogenicity is the targeting of quorum sensing (QS). Bacterial QS enables bacteria to coordinate their behaviour in response to changes in population density or external conditions [7–9]. It allows bacteria to regulate the expression of pathogenicity genes and coordinate collective actions as the production of virulence factors, biofilm formation, motility, and bacterial resistance through efflux pumps or production of antibiotic-modifying enzymes [10].

Bacteria release QS signal molecules called autoinducers, which accumulate and trigger specific responses once a threshold is reached. Gram-negative bacteria primarily employ N-acyl-L-homoserine lactones (AHLs) as autoinducers, whereas Gram-positive bacteria use autoinducing peptides (AIPs). The effect of autoinducers varies depending on the bacterial species and QS system [11,12]. Disrupting QS or bacterial cell-to-cell communication can have an antipathogenic effect since many systems affecting pathogenicity are controlled by QS. By interrupting this communication system, pathogenic bacteria can be rendered non-virulent [10,13,14]. Therefore, the aim of this study was to explore the potential anti-virulence activity of phenolic extracts from grape, pomegranate, and persimmon by-products against bacterial virulence activities, mainly modulated by QS, using reference bacterial strains.

2. Materials and methods

2.1. Chemicals and reagents

Bacteriological agar and bacterial culture media were purchased from Scharlab (Barcelona, Spain), as well as chloroform and ethanol (99%). Crystal violet (CV), HCl, and $MgCl_2$ were obtained from PanReac (Barcelona, Spain). Antibiotics, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), glucose, fluorescent stains, dimethyl sulfoxide (DMSO), triphenyl tetrazolium chloride (TTC), and phosphate-buffered saline (PBS) were obtained from Merck (Madrid, Spain). Streptomycin disks and glutaraldehyde (50%) were purchased from Thermo Fisher Scientific (Madrid, Spain). Ultrapure water was obtained from a Q-Gard® 1 system (Merck Millipore, Darmstadt, Germany) with a resistivity of 18.0 MΩ·cm.

2.2. Plant material and phenolic extracts

Grape, pomegranate, and persimmon by-products were processed at pilot scale in Mitra Sol Technologies S.L. throughout vacuum-expansion technologies [15] (see Supplementary Data). Grape by-product was obtained from the winemaking industry, whereas pomegranate and persimmon by-products were obtained from the juice industry. After processing, three phenolic extracts were obtained. Grape phenolic extract (GrPE) contained 15% of phenols with 13% of anthocyanins, pomegranate phenolic extract (PoPE) contained 40% of phenols with 30% of punicalagins, and persimmon phenolic extract (PePE) contained 5% of phenols with 13% of gallic acid. Stock solutions of extracts were prepared at 60 mg/mL (based in terms of total dried extract).

2.3. Bacterial strains

Most bacterial strains were obtained from Spanish Type Culture Collection (CECT). The use of different bacterial strains in this study was based on their suitability as models for specific assays. For instance, *Chromobacterium violaceum* CECT 494 and *Pseudomonas aeruginosa* CECT 4122 were chosen as QS models. *P. aeruginosa* was also selected for motility and efflux pump activity assessments. *Staphylococcus aureus* subsp. *aureus* CECT 59, *Pseudomonas putida* CECT 324, and the psychrotrophic enterotoxigenic *Bacillus cereus* INRA TZ415 strain (provided by Dr. Frédéric Carlin, Station de Technologie des Produits Végétaux, Institut National de la Recherche Agronomique, Avignon, France) were used in antibiofilm determinations. *Klebsiella pneumoniae* subsp. *pneumoniae* CECT 7787 was used to address β-lactamase inhibition. *Escherichia coli* CECT 515, *P. putida, K. pneumoniae, B. cereus*, and *S. aureus* were used to study the antibacterial mode of action of phenolic extracts.

Before each assay, the strains were routinely grown in their respective culture media and conditions to obtain fresh cultures (Supplementary Table S1). The optical density was measured at 600 nm (OD_{600}) to normalize the concentration of all bacterial suspensions to 0.5 ($\sim 10^8$ CFU/mL) [16–19], with a Cytation™ 3 Cell Imaging Multi-Mode reader (BioTek Instruments, Inc, Winooski, VT, USA). The OD at 760 nm (OD_{760}) was also determined in *C. violaceum* and *P. aeruginosa* suspensions [20,21].

2.4. Assessment of the antibacterial activity

Minimum inhibitory concentrations (MICs) and sub-inhibitory concentrations (SICs) of extracts were determined by broth microdilution method according to Clinical and Laboratory Standards Institute (CLSI) and elsewhere [22,23]. Extracts were two-fold serially diluted (15.00–0.03 mg/mL based in terms of total dried extract) in cation-adjusted Müller-Hinton medium and mixed with bacterial suspension (OD_{600} = 0.5) (200 µL as final volume per well). Cells with culture medium and antibiotic (erythromycin, kanamycin, or gentamycin) were included as positive controls and as abiotic control culture medium with extract and without bacteria. After 24 h at 37°C, the OD_{600} of plates was recorded to determine the percentage of inhibition at each dose. The OD_{760} was also recorded for *C. violaceum* and *P. aeruginosa*; similar inhibition percentages were obtained in both cases. The MIC in each case was confirmed by adding 10 µL of 0.5% TTC solution in each well. After 30 min at 37°C, the last well without red coloration was visually identified as MIC, and successive wells were considered SICs for further assays.

2.5. Anti-QS activity

2.5.1. Disk diffusion assay

QS inhibition in *C. violaceum* and *P. aeruginosa* exposed to GrPE, PoPE, and PePE were determined [24]. Pigment production by these strains was chosen as an indicator of bacterial QS activation [7]. Briefly, 100 µL of overnight LB grown cultures (OD_{600} = 0.5) of *C. violaceum* or *P. aeruginosa* were spread on LB agar plates. Immediately, 5-mm paper disks impregnated with MICs and SICs (1/2 × and 1/4 × MIC) of extracts were placed on the surface of agar. After 24 h at 37°C, the degree of inhibition was measured as the diameter (mm) of the uncoloured developed halo (absence of purple violacein for *C. violaceum* or blue active pyocyanin for *P. aeruginosa*) with cell growth. SIC of streptomycin (10 µg/mL) was experimentally determined and was used as control [25–27].

2.5.2. Violacein and pyocyanin production

To confirm the effects observed in the previous assay, quantification of violacein and pyocyanin was performed according to [28,29]. Overnight LB grown bacterial suspensions (OD_{600} = 0.5) were mixed with MICs and SICs (1/2 × to 1/128 × MIC) of extracts. Culture medium with extract and without bacteria (control) was included. After 24 h of incubation at 37°C, cells were pelleted for 10 min at 16 000 × *g*. Regarding violacein, the pellet was resuspended with DMSO, pelleted again, and supernatant OD was measured at 585 nm. *P. aeruginosa* supernatant was mixed with chloro-

form. The organic phase was acidified with 0.2 N HCl. The solution with pyocyanin turned into a reddish/pink colour, and its OD was measured at 520 nm. The percentage of both metabolites production was calculated by Eq. 1:

$$Metabolite\ production\ (\%) = \left(\frac{OD_{sample}}{OD_{control}}\right) \times\ 100 \qquad (1)$$

where OD_{sample} was the measure derived from extract-treated bacterial culture, and $OD_{control}$ was the measure of extract-untreated bacterial culture.

2.5.3. Metabolic profile of extracellular autoinducers by liquid chromatography-mass spectrometry (LC-MS/MS)

The autoinducer profile of *C. violaceum* and *P. aeruginosa* cultures (OD_{600} = 0.5) in LB broth supplemented with 1/2 × MIC of extract was determined. Autoinducers were extracted with ethyl acetate, evaporated, and resuspended in methanol as mentioned in [30].

A LCMS-8050™ system (Shimadzu, Kyoto, Japan) was used. The mobile phases, conditions, and gradient used for LC-MS/MS analysis are shown in Supplementary Table S2, and Supplementary Table S3 summarizes the MS parameter settings. Metabolomic identification of autoinducers in bacterial extracts was performed by untargeted Q3+ scan and targeted neutral loss scan based on the characteristic fragmentation patterns of the lactone ring at *m/z* 102.1 for AHLs [12]. Metabolite expression was quantified as the area under the curve formed during multiple reaction monitoring (MRM) of identified compounds. To observe changes in identified compounds, data were normalized to metabolites found in untreated cells and expressed as fold-change units. The MS parameters (precursor and product ions for MRM transitions, corresponding optimized collision energy, and retention time for each compound) are listed in Supplementary Table S4.

2.6. Antibiofilm assays

2.6.1. Antibiofilm activity

B. cereus, S. aureus, and *P. putida* strains were used for antibiofilm activity determinations since they produced the highest biomass formation compared to the rest of the strains used according to preliminary assays performed internally (in-house). Overnight LB grown bacterial suspensions (OD_{600} = 0.5) of *B. cereus, S. aureus*, and *P. putida* were incubated at 37°C for 4 h (initial biofilm formation), 24 h (preformed biofilm), and 48 h (mature biofilm). After first incubation, the extracts (two-fold serially diluted from 2 × to 1/64 × MIC) were added to the corresponding mature and forming biofilms (final volume of 200 µL). Untreated biofilms (control) were included for each determination. After 24 h of further incubation, planktonic cells were washed out with 1 × PBS, and biofilms were stained with 100 µL of 0.5% CV. After 20 min, CV was washed out with ultrapure water; then, 200 µL of ethanol was added to each well and measured OD_{630} immediately [31].

Antibiofilm activity was determined as follows (Eq. 2):

$$Biofilm\ inhibition\ (\%) = \left(\frac{OD_{control} - OD_{sample}}{OD_{control}}\right) \times\ 100 \qquad (2)$$

where $OD_{control}$ was the measure of untreated biofilm, and OD_{sample} was the measure derived from extract-treated biofilms. Antibiofilm concentration (AbC) of extract was considered to produce over 50% biofilm inhibition, destruction, or eradication [32].

2.6.2. Biofilm observation by field emission scanning electron microscopy (FESEM)

GrPE-treated forming biofilm (1/2 × MIC) and preformed biofilm (2 × MIC) of *P. putida* were replicated on coverslips in six-well plates. The coverslips with adhered cells were treated with 4% glutaraldehyde for 30 min and washed with 1 × PBS. Samples were dehydrated progressively with ethanol (30, 50, 70, 80, 90, and 100% for 15 min each) [33]. Micrographs of biofilms were recorded by Sigma 300 VP FESEM (Carl Zeiss, Germany) at 20 kV without coating.

2.7. Anti-efflux pumps activity

P. aeruginosa strain is suitable for assessing efflux pump activity because of its intrinsic multi-efflux pump systems [34]. Therefore, real-time quantification of energy-dependent efflux pump activity of *P. aeruginosa* was performed [35]. Briefly, overnight LB grown cultures were pelleted at 2500 × *g* for 5 min at 4°C, washed with 1 × PBS and 1 mM $MgCl_2$, and resuspended in 1 × PBS (OD_{600} = 2.0) for assays.

Bacterial solution was supplemented with the lipophilic stain with bright red fluorescence Nile Red (10 µM), incubated 2 h at 37°C and 100 rpm, and pelleted again. The pellet was resuspended in 1 × PBS with 1 mM $MgCl_2$ and mixed with MICs and SICs (1/2 × to 1/8 × MIC) of extracts. Nile Red hydrolysis was recorded at 560/655 nm of excitation/emission. Then, 2 mM of glucose were added to wells (final volume 200 µL) to efflux Nile Red. Immediately, efflux pump activity was monitored for 7 min. Glucose-treated/extract-untreated cells and glucose-untreated/extract-untreated cells were included as controls. Results were normalized to the fluorescence intensity before glucose addition. Nancy-520 fluorescent stain was also performed similarly to Nile Red. Cells were incubated with 10 µM of CCCP (15 min), further incubated with Nancy-520 (2 µM) (1 h at 37°C), pelleted at 2050 × *g*, and combined with extracts at indicated concentrations. Plates were monitored for 30 min at 520/554 nm of excitation/emission. Both stains fluoresce inside cells, and the fluorescence increase in cells was considered an indicator of efflux pump inhibition [35].

2.8. Bacterial motility

To visualize swimming and swarming motility disruption, *P. aeruginosa* was used for its well-known motility characteristics [36]. Briefly, a semisolid LB medium was formulated for swimming (0.25% of agar) and swarming (0.5% of agar) assessment [37]; also, the media was supplemented with 1/2 × MIC or 1/4 × MIC of extracts. In the centre of the plates, 1 µL of overnight LB grown bacterial suspension (OD_{600} = 0.5) was inoculated. The plates were incubated for 24 h, and then the motility of bacteria was imaged. The medium without extract was used as control.

2.9. β-Lactamase inhibition

K. pneumoniae strain was used for β-lactamase inhibition because of its high production of extended spectrum β-lactamase, which was verified by reagent-impregnated Beta Lactam™ disks (Thermo Fisher Scientific). Fresh cultures of *K. pneumoniae* (OD_{600} = 0.5) were mixed with SICs (1/2 × to 1/16 × MIC) of extracts. Untreated cells were included as control. After 24 h of incubation at 37°C, β-lactamase activity of treated and untreated cells was quantified using the β-lactamase activity assay kit from Merck, following manufacturer instructions.

2.10. Bacterial CMD assay

Bacterial CMD caused by extracts was assessed by CFDA-SE (carboxyfluorescein diacetate succinimidyl ester) stain [38]. Briefly, 100 µL (OD_{600} = 0.5) of the most sensitive Gram-positive and Gram-negative bacteria to each extract were mixed with its respective

MIC or 2 × MIC. Untreated cells were included as negative control. Kanamycin was used as positive control since it has been reported it causes CMD [39,40]. After 24 h of incubation, a CFDA-SE (0.4 µM) probe was added to each well, further incubated for 30 min, and the leakage of 5(6)-carboxyfluorescein succinidimyl ester (CFSE), a fluorescent molecule that accumulates inside bacterial cells and is released to the medium only if the cell membrane breaks, was measured at 490/520 nm of excitation/emission.

CMD was also assessed according to [41]. After 2 h of incubation of bacteria and extracts at mentioned doses, 30 µg/mL of propidium iodide (PI, a red-fluorescent nuclear and chromosome counterstain not permeant to live cells) was added to each solution. The cells were pelleted at 10 000 × *g* for 2 min at 4°C, washed with 1 × PBS, and stained with 1 µg/mL of Hoechst 33342 (Ho, a cell-permeant nuclear counterstain that emits blue fluorescence when bound to double-stranded deoxyribonucleic acid, dsDNA) for 15 min more. The cells were pelleted again and resuspended in 1 × PBS. Fluorescence of PI and Ho were recorded at 482/608 and 340/510 nm of excitation/emission, respectively. Percentage of damaged PI-stained bacteria was determined, and representative micrographs were also captured.

2.11. Statistical analysis

All experiments were performed in triplicate ($n = 3$), independently, and expressed as mean ± standard deviation (SD). GraphPad Prism 8.0.2 was used to perform the statistical analysis. Two-way analysis of variance (ANOVA) with Dunnett's post hoc tests were used. Results were considered of significance when $p < 0.05$.

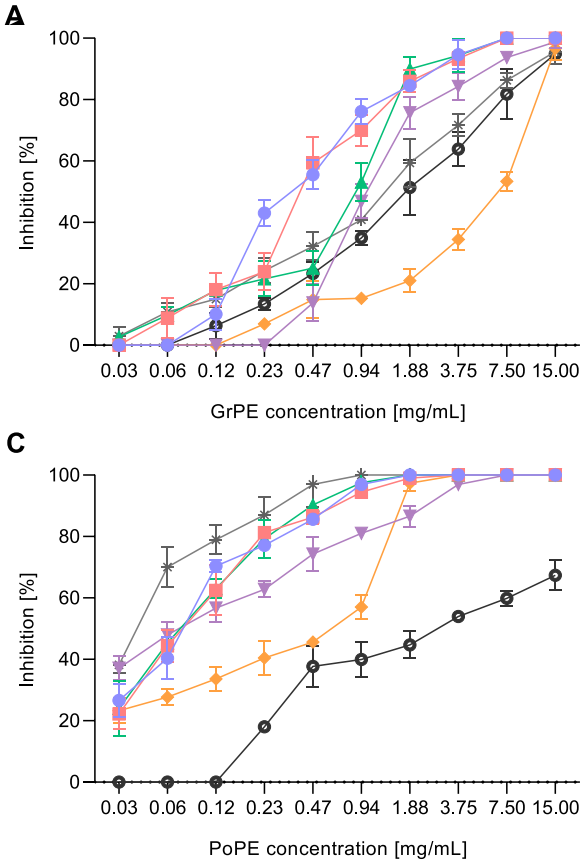
3. Results

3.1. Antimicrobial activity

Among the three extracts, PoPE (Fig. 1C) exhibited antibacterial activity at the lowest concentrations. The lowest MIC value of PoPE was against *P. putida* and *E. coli* (0.12 mg/mL extract or 0.05 mg/mL of phenolics), whereas the highest MIC value of PoPE was against *K. pneumoniae* at 15 mg/mL extract (6 mg/mL phenolics). GrPE (Fig. 1A) also showed a low MIC value as 1.88 mg/mL extract (0.28 mg/mL phenolics) against *P. putida*, while PePE (Fig. 1B) inhibited 70% of *K. pneumoniae* at 3.75 mg/mL extract (0.19 mg/mL phenolics). MICs of the extracts against all tested bacteria are shown in Fig. 1D. Concentrations below MIC were considered SICs for further assays. The overall tendency of antibacterial activity of extracts differed to the pattern observed with antibiotics. In Supplementary Fig. S1, bacteria showed antibiotic concentration-dependent sensitivity until a certain threshold, after which there was a drastic loss of effectiveness that could indicate antibiotic resistance potential. On the contrary, bacteria exhibited gradual adaptation or tolerance to lower concentrations of phenolic extracts.

3.2. Anti-QS activity

QS inhibition in *C. violaceum* and *P. aeruginosa*, by disk diffusion, was observed by PoPE, with results like streptomycin ($p >$



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	PoPE [mg/mL]		PePE [mg/mL]		GrPE [mg/mL]	
Microorganism	MIC	PC	MIC	PC	MIC	PC
B. cereus	0.23	0.09	1.88	0.09	3.75	0.56
C. violaceum	1.88	0.75	7.50	0.38	15.00	2.25
E. coli	0.12	0.05	7.50	0.38	15.00	2.25
K. pneumoniae	15.00	6.00	3.75	0.19	15.00	2.25
P. aeruginosa	1.88	0.75	7.50	0.38	15.00	2.25
P. putida	0.12	0.05	3.75	0.19	1.88	0.28
S. aureus	0.23	0.09	3.75	0.19	3.75	0.56

Fig. 1. Inhibitory activity of (A) grape phenolic extract (GrPE), (B) persimmon phenolic extract (PePE), and (C) pomegranate phenolic extract (PoPE) against bacterial pathogens. (D) Minimum inhibitory concentrations (MIC) of extracts and phenolic contents (PC) for tested bacterial strains. Bacterial inhibition is expressed as mean ($n = 3$) ± standard deviation (SD).

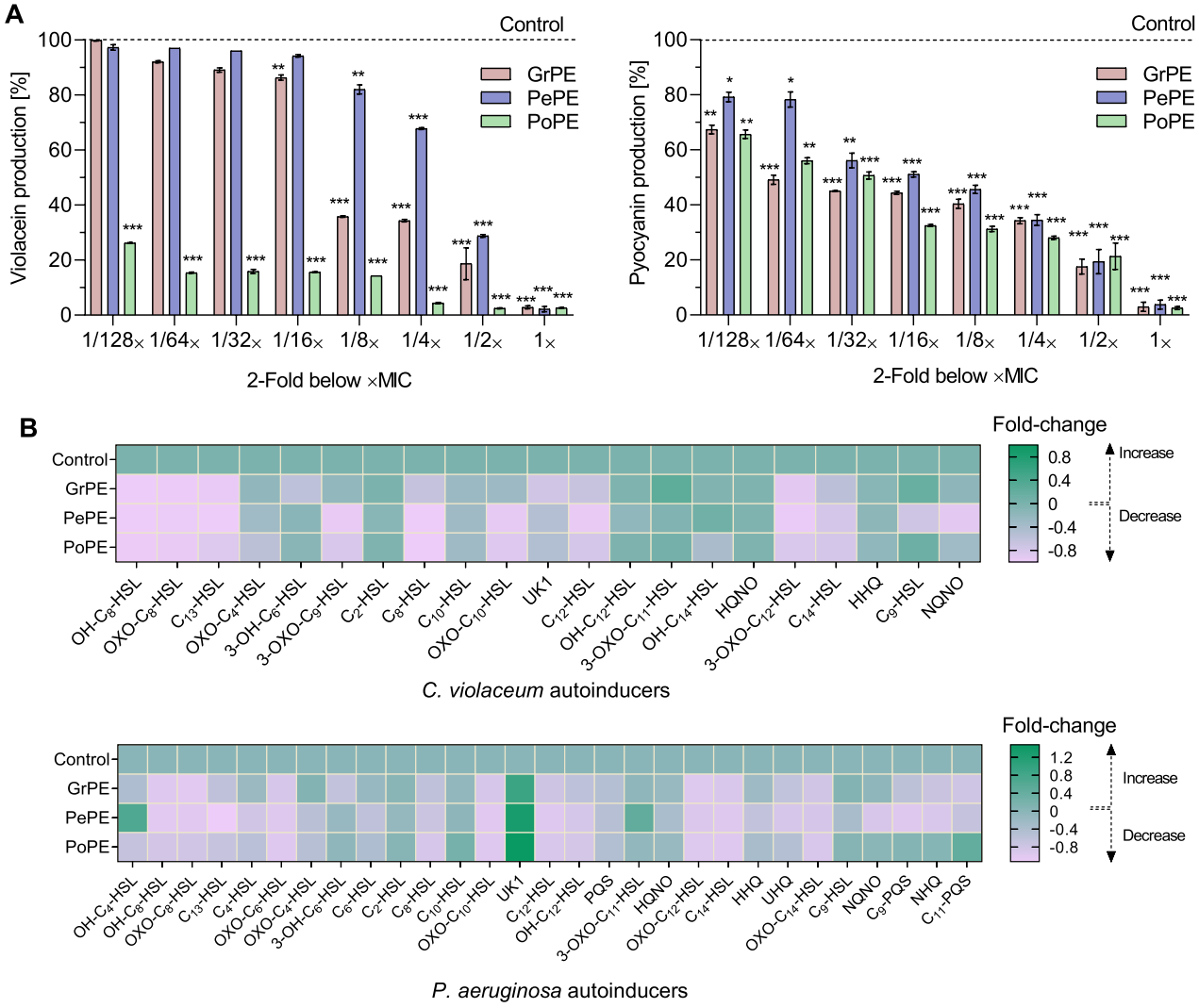

Fig. 2. Quorum sensing (QS) inhibition by grape phenolic extract (GrPE), persimmon phenolic extract (PePE), and pomegranate phenolic extract (PoPE). (A) Quantification of violacein and pyocyanin at minimum inhibitory concentration (MIC) and sub-inhibitory concentrations (SICs) of extracts. Overall, PoPE reduced the production of violacein and pyocyanin at the lowest SICs when compared to untreated cells (control) (***$p < 0.001$, **$p < 0.01$, *$p < 0.05$; two-way ANOVA with Dunnett's post hoc test). (B) Changes in extracellular autoinducers profile of untreated and 1/2 × MIC extract-treated cells of *C. violaceum* and *P. aeruginosa*. Values are expressed as mean ($n = 3$) of fold-change units.

0.05) (Supplementary Fig. S2C). QS inhibition diameters were also recorded by PePE at MIC, with unpigmented and isolated colonies of *C. violaceum* (Supplementary Fig. S2B); however, its effect was weaker than streptomycin ($p < 0.001$) (Supplementary Fig. S2C). GrPE also showed QS inhibition potential (Fig. S2C), but not as strong as the other samples. At SICs, extracts showed less QS inhibition, especially GrPE ($p < 0.001$), with inhibition halos similar to disk diameter (5 mm). Even though unpigmented colonies were observed within the zone of inhibition, the inherent pigmentation of phenolic extracts hindered the observation of a clear non-pigmented lawn surrounding the extract disk. Thus, QS-regulated metabolites violacein and pyocyanin were quantified to confirm anti-QS activity of extract samples.

Fig. 2 shows measurements of violacein and pyocyanin production due to QS bacterial activity. Violacein production was inhibited by all extracts. PoPE showed the highest reduction of this metabolite (<30%) even at the lowest concentration evaluated (1/128 × MIC) ($p < 0.001$). A similar reduction of violacein by PePE and GrPE at 1/2 × MIC and 1/8 × MIC, respectively, was observed ($p < 0.001$). Regarding pyocyanin, GrPE reduced 50% pyocyanin production at 1/32 × MIC ($p < 0.001$). PoPE and PePE also reduced pyocyanin production to values lower than 50% at concentrations of 1/16 × MIC and 1/8 × MIC, respectively. A low concentration of violacein and pyocyanin was determined in bacterial cultures grown in the presence of any one of the extracts at their respective MICs; the quantified pigments were considered metabolites initially produced by the basal cultures before the assay.

Since the extract samples reduced the production of violacein and pyocyanin, LC-MS/MS analysis was used to determine if these samples also modulated the profile of extracellular autoinducers in both bacterial strains as a potential anti-QS target. For instance, 18 AHLs were observed in *C. violaceum* (Supplementary Fig. S3) and 21 AHLs in *P. aeruginosa* (Supplementary Fig. S4). MS/MS analysis also showed the presence of three AQs (2-alkyl-4(1H)-quinolones) in *C. violaceum* and eight AQs in *P. aeruginosa*, resulting in a total of 21 and 29 autoinducers identified in *C. violaceum* and *P. aeruginosa*, respectively (Fig. 2B). After treatment at 1/2 × MIC, the analysis confirmed the modulation of QS by each phenolic extract (Fig. 2B). Thus, the concentration of 12 out of 21 autoinducers found in *C. violaceum* was reduced >0.5 fold-units by PePE, followed by PoPE (11 autoinducers) and GrPE (9 autoinducers). In

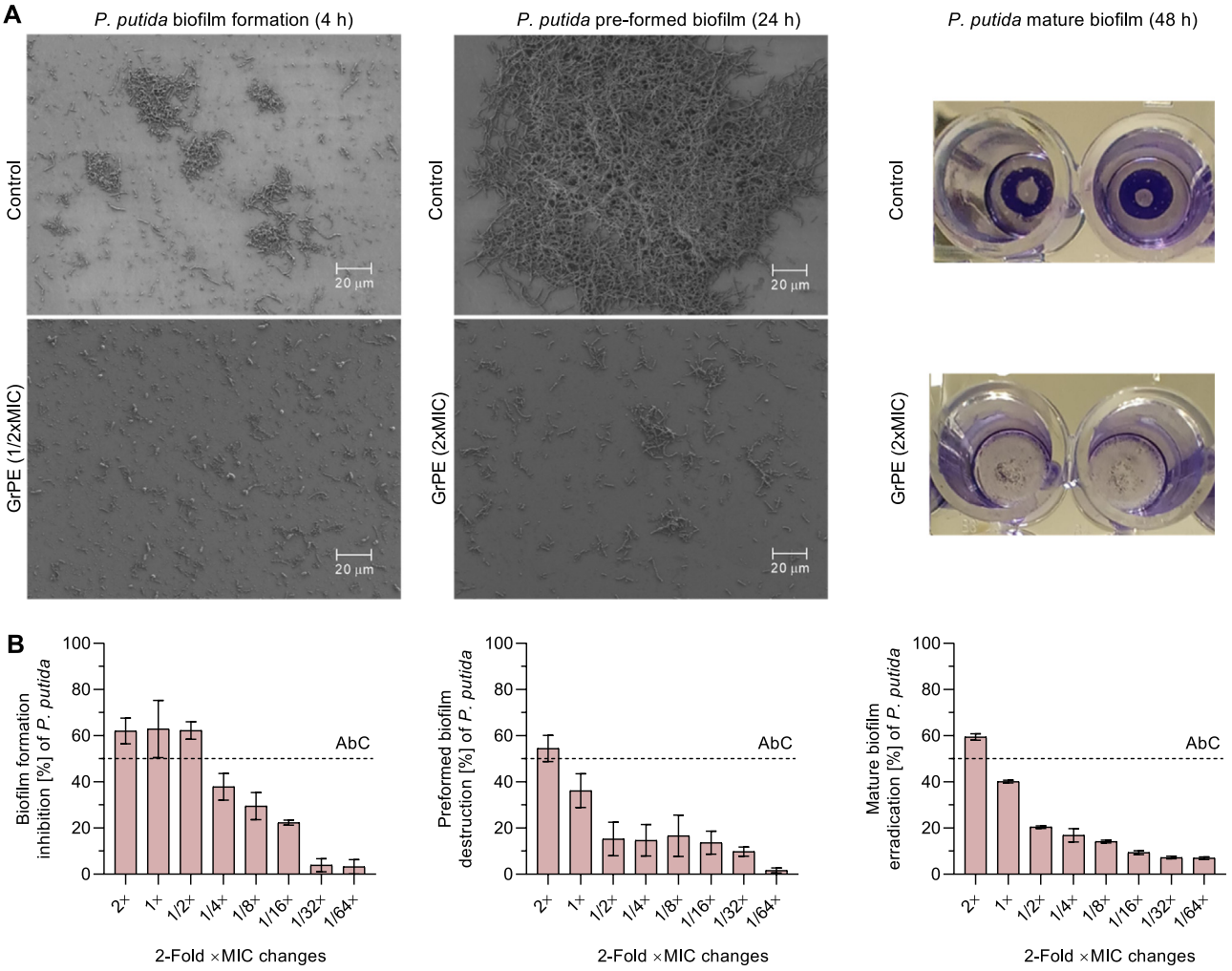

Fig. 3. Antibiofilm activity of grape phenolic extract (GrPE). (A) Field emission scanning electron microscopy (FESEM) micrographs of untreated and GrPE-treated *Pseudomonas putida* during their initial biofilm formation and preformed biofilm, as well as mature biofilm treated with crystal violet (CV). (B) Inhibition of initial biofilm formation, destruction of preformed biofilm, and erradication of mature biofilm of *P. putida*. Antibiofilm concentrations (AbC) that inhibited over 50% of biofilms were considered of significance. Values are expressed as mean (%, $n = 3$) ± standard deviation (SD).

P. aeruginosa, a similar pattern was observed with PePE, which reduced the concentration of 21 out of 29 autoinducers >0.5 fold-units, followed by GrPE (16 autoinducers) and PoPE (14 autoinducers). No changes in the degradation products of AHLs were detected between samples (MS/MS degradation pattern of 120.1>102.1).

In *C. violaceum*, C_{10}-HSL (decanoyl-L-homoserine lactone), 3-OXO-C_{12}-HSL (3-oxododecanoyl-L-homoserine lactone), 3-OXO-C_{11}-HSL (3-oxoundecanoyl-L-homoserine lactone), and C_9-HSL (nonanoyl-L-homoserine lactone) were the predominant autoinducers; among them, only 3-OXO-C_{12}-HSL was inhibited >0.5 fold-change units by all extracts, while C_9-HSL was additionally inhibited by PePE. In *P. aeruginosa*, AQs predominated, especially the *Pseudomonas* quinolone signal (PQS, 2-heptyl-3-hydroxy-4(1H)-quinolone), HQNO (2-heptyl-4-hydroxyquinoline N-oxide), HHQ (2-heptyl-4(1H)-hydroxyquinolone), NHQ (2-nonyl-4(1H)-hydroxyquinolone), and NQNO (2-nonyl-4-hydroxyquinoline *N*-oxide), while 3-OXO-C_{12}-HSL and C_4-HSL (butanoyl-L-homoserine lactone) were the most abundant AHLs. PePE reduced the content of PQS and HHQ >0.5 fold-change units, followed by PoPE and GrPE. 3-OXO-C_{12}-HSL was reduced by all the samples, while C_4-HSL was reduced only by PePE and PoPE. 3-OH-C_6-HSL (3-hydroxy-hexanoyl-L-homoserine lactone) was affected only by GrPE.

Interestingly, the phenolic extracts showed more effect against OXO-AHSLs in both bacteria, especially long tail autoinducers. At the same time, PePE and GrPE inhibited some AQs found in *P. aeruginosa*, while a modest increased in AQs content after exposure (Fig. 2B) to PoPE in *P. aeruginosa* was observed.

3.3. Antibiofilm activity

Results of antibiofilm activity of extracts are shown in Fig. 3 and Supplementary Fig. S5. GrPE exhibited strong antibiofilm effect since it inhibited the initial biofilm formation at 1/2 × MIC; it destroyed the preformed biofilm and eradicated the mature biofilm of *P. putida* at 2 × MIC. GrPE also destroyed biofilms of *S. aureus* and *B. cereus* at 2 × MIC (Supplementary Fig. S5). Fig. 3A exhibits FESEM-micrographs of untreated and GrPE-treated of initial biofilm formation and preformed biofilm of *P. putida*, as well as mature biofilms stained with CV (Fig. 3B). PePE did not show any antibiofilm effect, while PoPE eradicated mature *S. aureus* biofilm at 2 × MIC (data not shown).

3.4. Anti-efflux pumps activity

Fig. 4 shows that the three extracts tested blocked the activity of the efflux pumps of *P. aeruginosa*. All extracts reduced the

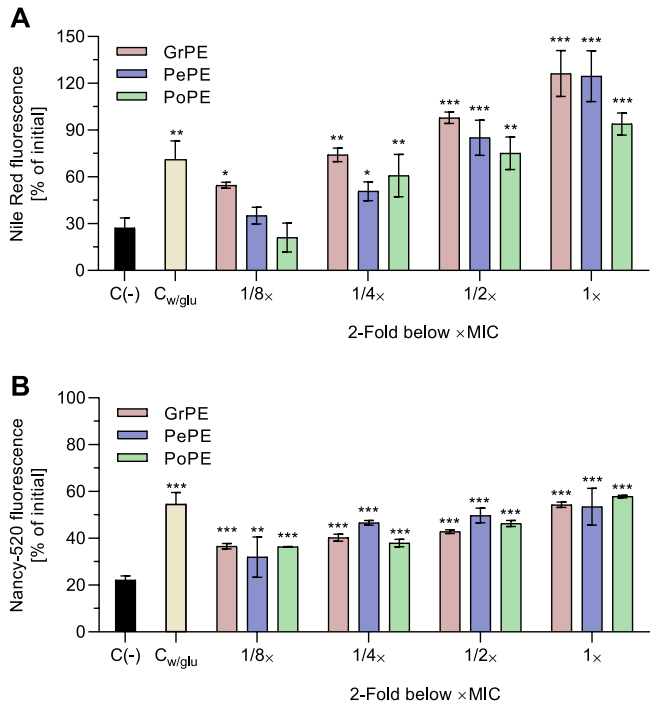

Fig. 4. Inhibition of *Pseudomonas aeruginosa* energy-dependent efflux pumps by Nile Red and Nancy-520 assays. Minimum inhibitory concentrations (MIC) and sub-inhibitory concentrations (SICs, 1/2 × to 1/8 × MIC) of grape phenolic extract (GrPE), persimmon phenolic extract (PePE), and pomegranate phenolic extract (PoPE) were compared to glucose-treated but extract-untreated cells (C-). Glucose-untreated and extract-untreated cells ($C_{w/glu}$) showed the efflux pumps of *P. aeruginosa* are energy dependent, maintaining both probes inside the cells, displaying high levels of fluorescence. After glucose addition, higher blockade of efflux pumps was observed with Nile Red probe than Nancy-520; even so, all extracts blocked the efflux of both probes in a dose-dependent manner when compared to (C-) ($^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$; two-way ANOVA with Dunnett's post hoc test). Results are represented as the mean (%, $n = 3$) ± standard deviation (SD).

efflux of Nile Red stain at high doses, which led to increasing its fluorescence over time even after glucose addition (Fig. 4A). GrPE retained 74% of Nile Red at 1/4 × MIC when compared to glucose-treated/extract-untreated cells ($p < 0.001$), which showed a minor fluoresce due to efflux of the stain. PePE and PoPE also reduced the efflux of Nile Red at 1/2 × MIC (85% and 75%, respectively) ($p < 0.001$). A similar effect was observed with Nancy-520 stain (Fig. 4B), where crescent concentrations of extracts retained the probe inside cells, traduced in a high fluorescence when compared to glucose-treated/extract-untreated cells ($p < 0.001$). GrPE and PoPE retained Nancy-520 in almost 40% at 1/8 × MIC ($p < 0.001$), while PePE retained the probe 50% at 1/4 × MIC ($p < 0.001$) when compared to glucose-treated/extract-untreated cells.

3.5. Disrupting bacterial motility

Swimming motility (Supplementary Fig. S6) was not inhibited by any of extracts. Regarding swarming motility, GrPE showed a strong motility disruption (++) at 1/2 × MIC, followed by a moderate effect of PoPe (+) and PePE (+), especially when compared to untreated cells.

3.6. Anti-β-lactamase activity

Extract-treated cells showed a reduction in the production of β-lactamase when compared to untreated cells (Fig. 5). PoPE-treated cells produced the least amount of β-lactamase, especially at 1/2 × MIC, reducing the enzyme in 88% (6.77 ± 0.28 mU/mL) ($p < 0.001$); moreover, its effect persisted from 1/4 × MIC to

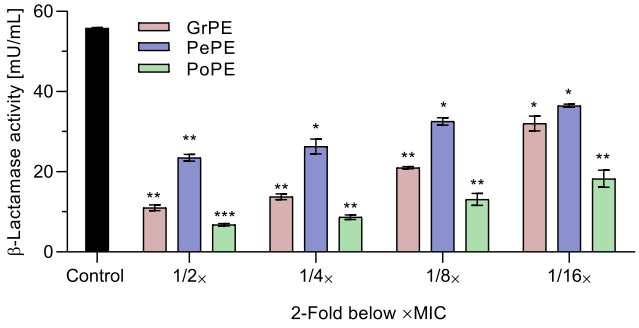

Fig. 5. Inhibition of β-lactamase activity (mU/mL) of *Klebsiella pneumoniae*. Sub-inhibitory concentrations (SICs, 1/2 × to 1/16 × MIC) of grape phenolic extract (GrPE), persimmon phenolic extract (PePE), and pomegranate phenolic extract (PoPE) were compared with untreated control. All extracts reduced the production of the enzyme, especially PoPE at 1/2 × MIC, which produced the least amount of β-lactamase among all samples ($^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$; two-way ANOVA with Dunnett's post hoc test). β-lactamase activity was expressed as mean ($n = 3$) ± standard deviation (SD).

1/8 × MIC until 64% of enzyme reduction ($p < 0.01$). GrPE also exhibited a noticeable reduction of 82% of β-lactamase activity at 1/2 × MIC (11.02 ± 0.74 mU/mL) ($p < 0.01$). PePE, at 1/2 × MIC, inhibited more than 50% of the enzymatic activity produced by *K. pneumoniae* ($p < 0.01$).

3.7. CMD effect of extracts

Gram-positive and Gram-negative strains that showed the highest inhibition at MIC values of each extract (Fig. 1) were used for this assay. According to the results (Fig. 6A), PoPE at MIC caused stronger CMD in *E. coli* and *B. cereus* than untreated cells ($p < 0.001$), with more than 0.5-fold-change units of CFSE leakage. The MIC of PePE also caused a high leakage of CFSE when compared to untreated *K. pneumoniae* and *S. aureus* ($p < 0.01$), in more than 0.5 and 0.75-fold-units. When the concentration of these extracts was increased at 2 × MIC, more distinctive effects were observed for PoPE ($p < 0.0001$) and PePE ($p < 0.001$), with more than 1 fold-change unit of CFSE leakage. As for GrPE, no significant differences were observed between untreated and MIC-treated *P. putida* and *B. cereus* bacterial strains ($p > 0.05$); however, at 2 × MIC of GrPE, a significant CFSE leakage increase was observed almost 0.25 fold-units ($p < 0.01$).

These results correlated with those obtained in the double staining assay PI/Ho (Fig. 6B). Weak CMD (20%) was observed in GrPE-treated *P. putida* and *B. cereus* bacteria at the two assessed doses ($p < 0.01$). The MIC of PePE caused moderate CMD in *K. pneumoniae* and *S. aureus* ($p < 0.01$), as well as a similar effect at 2 × MIC to kanamycin ($p < 0.001$). Fig. 6C shows a high percentage of 2 × MIC PePE-treated *K. pneumoniae* bacteria and PI-stained with CMD. Finally, PoPE at MIC also caused moderate CMD in *E. coli* and *B. cereus*, increasing when the concentration of the extract was 2 × MIC ($p < 0.001$).

4. Discussion

Phenolic compounds display antimicrobial activity against pathogenic bacteria; the cell membrane and enzymes are their primary targets [4]. In this study, phenolic extracts from three agroindustrial by-products showed a distinctive effect on bacterial QS of *C. violaceum* and *P. aeruginosa* strains. Thus, PoPE exhibited significant reduction in pyocyanin and violacein production at lower concentrations than the other tested extracts and those previously reported [42–44]. This reduction in pigment production can be attributed to the quenching of QS signalling, either by downregulating the synthesis of autoinducers, competing to bind receptor

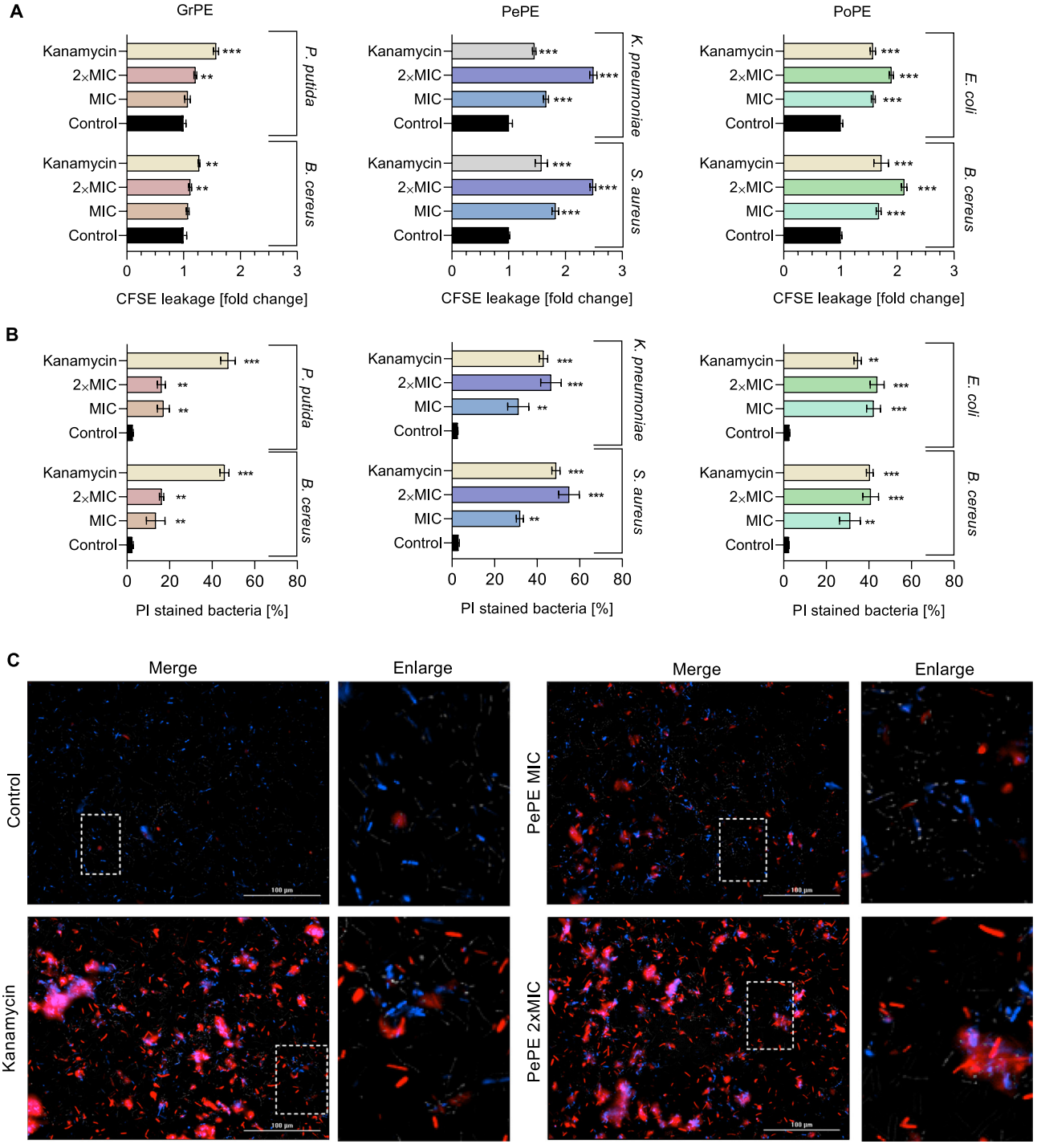

Fig. 6. Cell membrane damage (CMD) of bacteria after exposure to grape phenolic extract (GrPE), persimmon phenolic extract (PePE), and pomegranate phenolic extract (PoPE). (A) Leakage of 5(6)-carboxyfluorescein succinidimyl ester (CFSE) fluorescent stain from bacterial cells at minimum inhibitory concentration (MIC) and 2 × MIC. PePE and PoPE caused noted CMD in bacterial cells (****$p < 0.0001$, ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$, two-way ANOVA with Dunnett's post hoc test). Data are shown as fold-change units ($n = 3$) ± standard deviation (SD). (B) Propidium iodide (PI) stained bacteria due to CMD after extract exposure. PePE at 2 × MIC caused similar CMD than kanamycin in *Klebsiella pneumoniae* (***$p < 0.001$, **$p < 0.01$; two-way ANOVA with Dunnett's post hoc test). Data are shown as percentage of PI-stained bacteria ($n = 3$) ± standard deviation (SD). (C) Representative merge membrane damage micrographs of PePE-treated *K. pneumoniae*. Blue-emitting fluorescent Hoechst 33342 (Ho) was used to stain all cells, while red-emitting fluorescent PI was used to identify dead cells due to CMD.

proteins, or enzymatically degrading autoinducers [44]. In particular, the extracts altered the metabolomic profile of autoinducers at 1/2 × MIC. Although degradation patterns of autoinducers were not analysed, it might be assumed that phenolic extracts could inhibit autoinducer synthesis or act as analogues of QS signal molecules.

This study highlights the disruptive effects of phenolic extracts on bacterial QS system and the attenuation of virulence activities. Each extract showed specific modulation of autoinducer synthesis, suggesting targeted effects on different bacterial populations. Particularly the reduction of short-chain autoinducers (C_4-HSL – C_8-HSL (octanoyl-L-homoserine lactone)) by PoPE in *C. violaceum* and

P. aeruginosa aligns with previous reports of pomegranate extract ability to reduce violacein production [42–45], indicating a potent anti-QS activity or quorum quenching (QQ). An inverse relationship between the phenolic extracts, PQS, and pyocyanin concentration was observed, explaining the determined reduction in pyocyanin production [46]. Among the extracts, PePE showed a noted blocking effect on AHL and AQ synthesis which are involved in the activation of other autoinducers and regulation of virulence genes [12,46]; however, because of its low phenolic content, its activity was not as pronounced as that of PoPE and GrPE.

Furthermore, our study showed all extracts downregulated autoinducers associated with biofilm formation, such as C_4-HSL, C_6-HSL (hexanoyl-L-homoserine lactone), C_8-HSL, C_{12}-HSL (dodecanoyl-L-homoserine lactone), PQS, and 3-OXO-C_{12}-HSL [9,47]. Interestingly, only GrPE exhibited antibiofilm activity, which is probably by reducing OXO-C_8-HSL (oxooctanoyl-L-homoserine lactone), PQS, and particularly 3-OH-C_6-HSL [48]. Since PoPE only showed an effect against the mature biofilm of *S. aureus*; the observed effect might not depend on bacterial QS but on reactive oxygen species (ROS) that promote biofilm development [49], which would show the differences between the extract samples. The effect of GrPE against the initial formation of *P. putida* biofilms was independent of bacterial growth inhibition but dependent on the anti-QS activity of the extract. In contrast, the destruction and eradication of the preformed and mature biofilms of *P. putida, B. cereus*, and *S. aureus* by GrPE depended on other activities of the extract such as antioxidant and antibacterial [31] activities in addition to anti-QS activity.

GrPE also exhibited a noticeable effect on disrupting bacterial motility, particularly swarming motility, whereas PePE and PoPE showed a modest effect. Swarming motility, which relies on flagellar movement, is highly regulated by QS expression [36], and it requires raft formation and surfactant production to reduce surface tension. Hence, swarming motility disrupting in *P. aeruginosa* aligns with anti-QS activity of extracts. On the contrary, extracts did not affect swimming motility in *P. aeruginosa* since it is based on individual movement in liquid powered by rotating flagella, suggesting that the activity of these extracts is not directed at flagella [36]. The regulation of C_4-HSL, C_6-HSL, and OXO-C_6-HSL (oxohexanoyl-L-homoserine lactone) may contribute to quenching swarming in bacteria [37]. Furthermore, reduction of the OH-C_6-HSL autoinducer could serve as a target to block bacterial motility and disrupt biofilms.

QS plays a role in activating antimicrobial resistance pathways in bacteria such as efflux pumps and the production of antimicrobial-modifying enzymes [10]. This study also emphasizes the potential of phenolic extracts to inhibit energy-dependant efflux pumps in *P. aeruginosa* and β-lactamase activity in *K. pneumoniae*, in a dose-dependent manner. Efflux pumps in bacteria are responsible for transporting antimicrobial molecules out of the cell [50], while multidrug-resistant (MDR) bacteria produce antimicrobial-modifying enzymes, such as β-lactamases to inactivate antimicrobial molecules [5]. Additionally, autoinducers control the expression of ATP-binding cassette or ABC transporters [51]. Results indicate that phenolic extracts interfere with the activation pathway of ABC transporter systems, which depend on ATP hydrolysis for the influx or efflux of substrates [35].

It has been suggested that the modulation of long tail HSL autoinducers may be involved in the blockade of the ABC efflux pumps of *P. aeruginosa* [52], specifically 3-OXO-C_{12}-HSL and OXO-C_{14}-HSL (oxotetradecanoyl-L-homoserine lactone), that diffuse via ABC transporters into and out of the cell. Given that ABC transporters are located in cell membranes [51], chemicals that disrupt the inner bacterial membrane may indirectly inhibit efflux as demonstrated by the assays of CMD caused by the tested phenolic extracts. On the other hand, the decrease in OXO-C_6-HSL autoinducer by the extracts could influence the reduction of β-lactamase activity in *K. pneumoniae*. This is in line with a report suggesting that OXO-C_6-HSL also influences β-lactamase production, particularly when complexed with the LuxR activator protein for the PluxI promoter (LuxI/LuxR QS system), allowing bacteria to survive in the presence of high concentrations of β-lactams [5]. The results highlighted the need to investigate the direct role that bacterial QS regulation plays in β-lactamase genes expression, further expanding the potential of phenolic extracts in combating MDR bacteria.

Each extract displayed distinct modulatory effects on various virulence factors, reflecting their inherent compositions. PePE and PoPE induced the leakage of cellular content as confirmed by CFDA-SE and IP/Ho assays, consistent with the mode of action of phenolic compounds [4]. GrPE exhibited a different mechanism to inhibit bacterial proliferation, possibly targeting enzymes such as dehydratase or DNA gyrase [53], in addition to causing CMD. CFDA-SE assay results for GrPE suggest CFSE was evenly distributed inside cells as well as freely available [38]. The IP/Ho assay also indicated CMD, as IP selectively entered cells with compromised plasma membranes.

In conclusion, this study underscores the potential of phenolic extracts, namely GrPE, PePE, and PoPE, in modulating various and specific bacterial virulence activities through the inhibition of extracellular autoinducers and QS expression. Moreover, results support the application of these extracts in novel antimicrobial strategies, such as the development of antibiotic adjuvants or the resensitization of antibiotic-resistant bacteria. The search for phenolic compounds with targeted anti-virulence activities contributes to the advancement of health and science, especially in various areas of biochemistry and industry.

Declarations

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Competing interests: The authors declare that they have no conflicts of interest related to this work.

Ethical approval: Not required.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ijantimicag.2023.106937.

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