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Original Article

Natural products and polysorbates: Potential Inhibitors of biofilm formation in *Pseudomonas aeruginosa*

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Abstract

Introduction: With all the challenges super bugs are imposing, biofilm formation opens the door against various more complicated challenges. Such issue may be highlighted with the ability of the latter to render the antibiotics hardly accessible to bacterial cells and sheds the light on the importance of finding antibiofilm formers. Therefore, we assessed the inhibitory effect of natural product extracts (ginger, wild blueberry) and polysorbates (PS20, PS80) on biofilm formation at the molecular level.

Methodology: Growth inhibition assay was performed to test the effect of ginger (*Zingiber Officinale*), wild blueberry (*Vaccinium Angustifolium*), and polysorbates on *Pseudomonas aeruginosa* (PAN14) growth. Transcription levels of biofilm exopolysaccharides encoding genes (*ndvB*, *pelC*, *algC*) and quorum sensing genes (*lasI*, *lasR*, *rhlI*, *rhlR*) for LasI/LasR and RhlI/ RhlR systems were evaluated by RT qPCR.

Results: The polysorbates and the extracts of both ginger and wild blueberry had no effect on the growth of *P. aeruginosa*. Biofilms' examination has unraveled the effectiveness of treatments used in reducing its formation. Moreover, a significant reduction in the expression of all genes tested for biofilm exopolysaccharides and its quorum sensing system was observed.

Conclusion: The decrease in the relative gene expression of the exopolysaccharides and quorum sensing encoding genes sheds the light on the mechanism of action of ginger and wild blueberry's constituents as well as polysorbates 20 and 80 on *P. aeruginosa* biofilm formation. Future studies need to assess the antibiofilm effect of each fraction of herbal extracts separately.

Key words: biofilm inhibition; *Pseudomonas aeruginosa*; exopolysaccharides; quorum sensing; gene expression.

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Introduction

Pseudomonas aeruginosa is an opportunistic bacterial pathogen that causes infections in immunocompromised, as well as immunocompetent hosts [1]. These include, skin, urinary tract, blood, wound, ear, and eye infections [2]. *P. aeruginosa* is characterized by its high ability to produce biofilms, making it a hard pathogen to treat and a powerful model for biofilm studies [3,4]. The biofilm's hallmark is enabling the bacteria within to adhere on living and nonliving surfaces as well as rendering the bacteria resistant to antibiotics and immune defense [5,6]. This resistance is due to the genetic alterations within the biofilm as a result of chronic and persistent infections, which in-turn further complicates the eradication of *P. aeruginosa* infections [5,7].

Cells within *P. aeruginosa* biofilm are surrounded by a shield of extracellular polymeric substances (EPSs) [8]. EPSs consist mainly of exopolysaccharides such as, Pel, alginate, as well as1, 3-β-d-Glucans whose expression favours biofilm formation and renders the antimicrobial agents hardly accessible to bacterial cells, and the immune defense clearance mechanisms ineffective [9–11]. Moreover, Psl is considered one of the major exopolysaccharides in the *P. aeruginosa* biofilm matrix, it plays a role in providing a first line of defense during the initial stages of biofilm formation against wide range of antibiotics [12]. At the molecular level, the pel gene operon cluster is essential for pellicle formation in which *pelC* gene encodes for a glycosyltransferase that plays a role in polysaccharides migration [10,13]. Alginate is a major component of *P. aeruginosa* exopolysaccharides whereby a phosphomannomutase, encoded by *algC* gene, is responsible for its biosynthesis [14,15]. Moreover, in *P. aeruginosa*, the cyclic-β-(1,3)-glucans are secreted to

the extracellular matrix within a biofilm and a glucosyltransferase, encoded by the *ndvB* gene, is responsible for their synthesis [16,17].

Nonetheless, cells within the biofilm communicate through a quorum sensing (QS) system [18]. QS is a cell-density dependent mechanism through which bacteria in a group coordinate their behaviors in response to signals known as auto-inducers [18,19]. In addition, bacterial behaviors such as virulence factors production and biofilm formation are controlled by the QS system [20]. In *P. aeruginosa*, the acetylated homoserine lactones (AHLs) are the auto-inducers of two major QS systems: LasI/LasR and RhlI/RhlR, whereby 3-oxo-C12-HSL (OdDHL) and C4-HSL (BHL) are their corresponding AHLs respectively [19,21]. Studies have shown that knocking out the genes encoding for QS auto-inducers resulted in the formation of thin-layered biofilms [21].

As a part of targeting biofilms, herbal extracts were used as they are generally safe, and 85% of traditional medicine was derived from them [22,23]. The fact that molecules derived from herbal extracts do not directly affect the growth of bacteria made them interesting antibiofilm structures [24]. Ginger (*Zingiber Officinale*) and wild blueberry (*Vaccinium Angustifolium*) extracts are known to have plenty of phenolic compounds that can have antibiofilm properties, such as gingerols, proanthocyanins, and condensed tannins [25,26]. The alkyl chain present in some phenols and polyphenols can provide a binding specificity of the molecule to certain receptors/enzymes, and the phenol ring can play a role in the agonistic or antagonistic properties [20]. In a study done by Karuppiah *et al*. (2011) aiming to test the antibacterial activity of ginger rhizomes ethanolic extracts on the isolates of Gram-negative organisms and Gram-positive. The ginger rhizomes ethanolic extracts demonstrated antibacterial activity against five clinical isolates with zone of growth inhibition ranging from 4 mm to 16 mm. The maximum zone of inhibition was showed against Bacillus sp. (16.55 mm) followed by *E. coli* (15.50 mm) and *P.aeruginosa* (14.45 mm) [27].

In addition to assessing the antibiofilm effect of herbal extracts, polysorbates 20 (PS20) and 80 (PS80) or polyoxyethelene sorbitan monolaurate, and polyoxyethelene sorbitan monooleate respectively were also used. PS20 and PS80 are non- ionic surfactants and emulsifiers that are used as food additives and as a constituent in various pharmaceutical combinations [28,29]. Moreover, PS20 and PS80 are tolerated in humans up to 1% according to the Center for Food Safety and Applied Nutrition, U.S. Food and Drug Administration [30]. Previous study has shown the antibiofilm property properties of both PS20 and PS80 on *E.coli* O104:H4 [29].

With the advances in molecular techniques, such methods became important to validate a phenotypic result. RT-qPCR helps in the detection of the gene expression levels. This method will help determine the direct effect of the plants extracts or any other inhibitor on biofilm formation, by determining the level of expression of three exopolysaccharide genes (*ndvB*, *pelC*, *algC*) and four quorum sensing genes (*lasI*, *lasR*, *rhlR*, *rhlR*) that are essential in biofilm formation in *P. aeruginosa.*

The aim of the study was to assess the potential ability of ginger, wild blueberry extracts, and polysorbates 20 and 80 to inhibit biofilm formation in *P. aeruginosa in-vitro*, and to determine the mechanism of action behind the activity, and the relative expression of the genes required in the process.

Methodology

Source of Bacterial Isolate

The *P. aeruginosa*, strong biofilm-forming isolate, used in the study (PAN14) was formerly obtained by deep tracheal aspirate (DTA) from a patient with nosocomial infection [10]. Isolate's identification was done through colonial morphology, pigment production, grape-smell, API20 NE kits (bioMerieux. SA 69820, Marcy l'Etoile, France) and tested for susceptibility by disc diffusion method against a set of antimicrobial agents according to Clinical and Laboratory Standards Institute (CLSI) guidelines [31].

Source of Extracts, PS20 and PS80

Ginger roots (China), and wild Blueberry (Chile) were obtained from a local market in Beirut, Lebanon. Polysorbate 20 **(**Polyoxyethylene sorbitan monolaurate) and Polysorbate 80 (Polyoxyethylene sorbitan monooleate) were obtained from Sigma Aldrich (Sigma Chemical Co., St. Louis, MO, USA).

Preparation of extracts

Ginger extract

32 g of fresh ginger were blended with 70 mL ethanol. The sample was then kept at room temperature for 24 hours followed by vortexing for 15 minutes. Afterwards, soaked ginger was filtered through a sterile syringe filter of 0.2 µm and stored at -20ºC. The filtrate obtained was used to test its effect on *P. aeruginosa* biofilm formation.

Wild blueberry extract

The extraction of wild blueberry was performed according to Vattem *et al.* [32]. Two different approaches were followed where the first sample was prepared from peel and pulp and the second was prepared from pulp only. For both samples, 0.53 g of wild blueberry was weighed using a precision balance and suspended in 10 mL distilled water. The suspensions were stirred on a magnetic stirrer for 15 minutes. Then, the mixtures were added to tubes and centrifuged at 3000 rpm for 10 minutes at 10ºC. Finally, supernatant was filtered through a sterile syringe filter of 0.2 µm and stored at 4ºC. The mixture obtained was used to test its effect on *P. aeruginosa* biofilm formation.

Growth inhibition assessment

Overnight culture of PAN14 was diluted 1:100 in fresh Luria broth (LB). Different concentrations of ginger (1%, 3%, 5%, 10% in sterile water), wild blueberry (15%, 20%, 25% in sterile water), PS20 (0.1%, 0.2%, 0.3% in sterile water), and PS80 (0.1%, 0.2%, 0.25% in sterile water) were added to the bacterial suspension. Then, tubes were incubated in the shaking incubator (170 rpm) at 37ºC for around 7 hours, during which OD was measured using a densitometer (WPA CO 8000 Biowave Cell Density Meter, Cambridge, UK) at 600 nm each half an hour to track bacterial growth. The tubes were then kept overnight at 37ºC and OD was measured the next day**.**

Biofilm formation assessment using microtiter plate assay

The effect of different ginger, wild blueberry, and polysorbates' concentrations on PAN14 biofilm formation was assessed in a microtiter plate as described by Merrit *et al.* (2011) with minor modifications [33]. Briefly, an overnight culture of PAN14 was adjusted to 1 McFarland and further diluted 1:50 in fresh LB broth. After this, 100 µL of bacterial suspension was added in sextuplicate into the microtiter plate and considered as the untreated positive control. The different concentrations of ginger, wild blueberry, PS20, and PS80, mentioned earlier, were added to the bacterial suspension as given in (Table 1). The plates were stained with 1% crystal violet and then eluted with ethanol. Using BIO-TEK EL×800 Automated Microplate Reader, absorbance was measured in wells at 630 nm. Student's t-test was performed and a p-value < 0.05 was considered statistically significant. Biofilms that were significantly reduced (p-value < 0.05) upon addition of different treatments' concentrations were subjected to RNA extraction from a duplicate microtiter plate for RT-qPCR.

Confocal Laser Scanning Microscopy (CLSM)

Biofilms architecture and structural integrity were examined through Zeiss LSM 710 Confocal microscopy (Germany) and visualized with the 100x oil immersion lens. All treated and untreated biofilms were grown on Whatman filter paper (0.2 µm pore size). The protocol for imaging biofilms was performed as described previously [34], with significant modifications. Briefly, an overnight PAN14 liquid culture was adjusted to 10 mL of 10^8 CFU. Each of the treatments, 5% ginger, 25% wild blueberry peel and pulp, 25% wild blueberry pulp, 0.2% PS20, and 0.25% PS80 were added to the culture tubes and then deposited on filter membranes under negative pressure using a vacuum pump. Whatman filter papers were then transferred to LB agar plates and incubated at 37ºC for 48 hours. Filter papers were cut to small pieces and fixed overnight with 3.7% (w/v) formaldehyde. DAPI, for nucleic acid staining (Sigma Aldrich, St. Louis,

Table 1. Experimental design of the five microtiter plates.

Plate Number	Plate Content
First plate	NC
	PC (PAN14)
	PAN14 + 1% ginger
	PAN14 + 3% ginger
	PAN14 + 5% ginger
	PAN14 + 10% ginger
Second plate	NC
	PC (PAN14)
	PAN14 + 15% wild blueberry (pulp)
	PAN14 + 20% wild blueberry (pulp)
	PAN14 + 25% wild blueberry (pulp)
Third plate	NC
	PC (PAN14)
	PAN14 + 15% wild blueberry(peel and pulp)
	PAN14 + 20% wild blueberry(peel and pulp)
	PAN14 + 25% wild blueberry(peel and pulp)
Fourth plate	NC
	PC (PAN14)
	PAN14 + 0.1% PS20
	PAN14 + 0.2% PS20
	PAN14 + 0.3% PS20
Fifth plate	NC
	PC (PAN14)
	PAN14 + 0.1% PS80
	PAN14 + 0.2% PS80
	PAN14 + 0.25% PS80

NC: Negative Control; PC: Positive Control; PAN14: *Pseudomonas aeruginosa* PAN1.

Table 2. Exopolysaccharides, quorum sensing, and housekeeping gene primers and the corresponding annealing temperatures.

Genes	Primers Sequences	Product size	Annealing Temperature
ndvB	**F:**5'-GGCCTGAACATCTTCTTCACC-3' **R:**5'-GATCTTGCCGACCTTGAAGAC -3'	138bp	65ºC
algC	**F:** 5'-CTACTTCAAGCAGATCCGC-3' **R:**5'-AGGTCCTTCAGGTTCTCC-3'	204bp	65ºC
pelC	**F:** 5'-TGCTCCAGCTTCACCAG-3' **R:** 5'-CAGTTGCAGGTCGCCTT-3'	192bp	60ºC
lasI	**F:**5'-CTACAGCCTGCAGAACGACA-3' **R:**5'-ATCTGGGTCTTGGCATTGAG-3'	168bp	65ºC
lasR	**F:**5'-ACGCTCAAGTGGAAAATTGG-3' **R:**5'-GTAGATGGACGGTTCCCAGA-3'	274bp	65ºC
rhlI	**F:**5'-CGAATTGCTCTCTGAATCGCT-3' **R:**5'-GGCTCATGGCGACGATGTA-3'	182bp	65ºC
rhlR	**F:** 5'-AGGAATGACGGAGGCTTTTT-3' **R:** 5'- CCCGTAGTTCTGCATCTGGT-3'	231bp	60ºC
rpoD	**F:** 5'-AGGTGGCGTAGGTGGAGAA-3' **R:** 5'-GGGCGAAGAAGGAAATGGTC-3'	177bp	65ºC

F: Forward; R: Reverse.

United States), was diluted 1:1000 and added to the samples for 1 hour at room temperature and away from light. Under similar conditions, Calcofluor white Stain M2R (1 g/L, Sigma Aldrich, St. Louis, United States) was diluted (100 µL/1 mL PBS) and added to stain the polysaccharide component of the biofilm matrix. Each of the above steps was followed by a washing step with PBS to prevent excessive staining. Samples were kept wet and then visualized with CLSM on confocal microscopy petri dishes.

RNA extraction

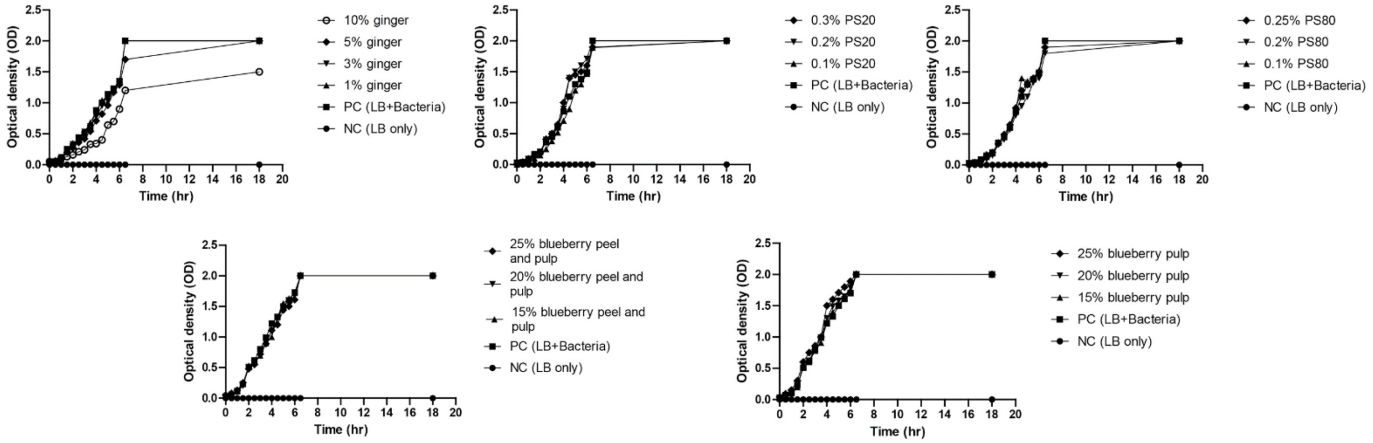
In accordance with the manufacturer's procedure of PowerBiofilm RNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad USA), RNA extraction was done on crude extracts and polysorbates-treated and untreated bacterial cells in microtiter plate wells. Biofilms were scrapped from the walls of the microtiter plate and added to 1.5 mL of fresh LB broth in a 2 mL microcentrifuge tubes. Tubes were centrifuged and pellet was treated with appropriate buffers then incubated with 50 µL DNase (10 U).

Reverse transcription PCR (RT-PCR) and quantitative reverse transcription PCR (RT-qPCR)

After obtaining RNA, reverse transcription and cDNA synthesis were done using QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany) according to manufacturer's protocol. RNA concentration of 0.1 µg, for each sample, was calculated for cDNA synthesis.

RT-qPCR was carried out on the untreated sample (positive control, PAN14), as well as samples treated with ginger and blueberry extracts and polysorbates whose concentrations have shown the highest anti-biofilm effect and that were considered statistically significant (p-value < 0.05). The effectiveness of the treatments used was assessed through RT-qPCR to detect the expression level of three exopolysaccharide genes (*ndvB*, *pelC*, *algC*) and four quorum sensing

Figure 1. Growth curves of PAN14 with ginger extract, wild blueberry extract, and polysorbates at different concentrations.



genes (*lasI*, *lasR*, *rhlR*, *rhlR*) that are essential in biofilm formation in *P. aeruginosa*. The housekeeping gene used was *rpoD*, which encodes σ^{70} of *P. fluorescens* and that shows 96.2% similarity with 83.3% identity with *P. aeruginosa* rpoD [35,36]. Sequences primers are presented in (Table 2). The Minimum Information for Publication of Quantitative Reverse Transcription PCR Experiments (MIQE) guidelines were followed for applying RT-qPCR. Finally, SDs were calculated and student's t-test was performed in which a p-value < 0.05 was considered statistically significant.

Results

Effect of ginger wild blueberry and polysorbates 20 and 80 on Pseudomonas aeruginosa growth

Results suggest that ginger, wild blueberry pulp, and wild blueberry peel and pulp at concentrations ranging from 1% to 5%, and 15% to 25% (for both blueberry extracts), respectively, did not have a significant impact on PAN14 growth. Similarly, PS20 and PS80 have shown no effect on the growth at concentrations ranging from 0.1% to 0.3% or 0.25%, respectively (Figure 1).

Effect of ginger, wild blueberry and polysorbates on Pseudomonas aeruginosa biofilm formation

A statistically significant reduction (p-value < 0.05) in the biofilm formation was observed in the experimental group (i.e. cultures treated with 5% ginger, 25% wild blueberry (*Vaccinium Angustifolium*), 0.3% PS20, 0.25% PS80) in comparative with the positive control (untreated PAN14) according to measured optical densities. Biofilms treated with 5% ginger, 25% wild blueberry pulp and 25% wild blueberry peel with pulp, 0.3% PS20, 0.25% PS80 were decreased by 42.47%, 36.5%, 40.68%, 36.5%, 63.7%, respectively (Table 3).

Visualizing the Biofilm with Confocal Laser Scanning Microscopy (CLSM)

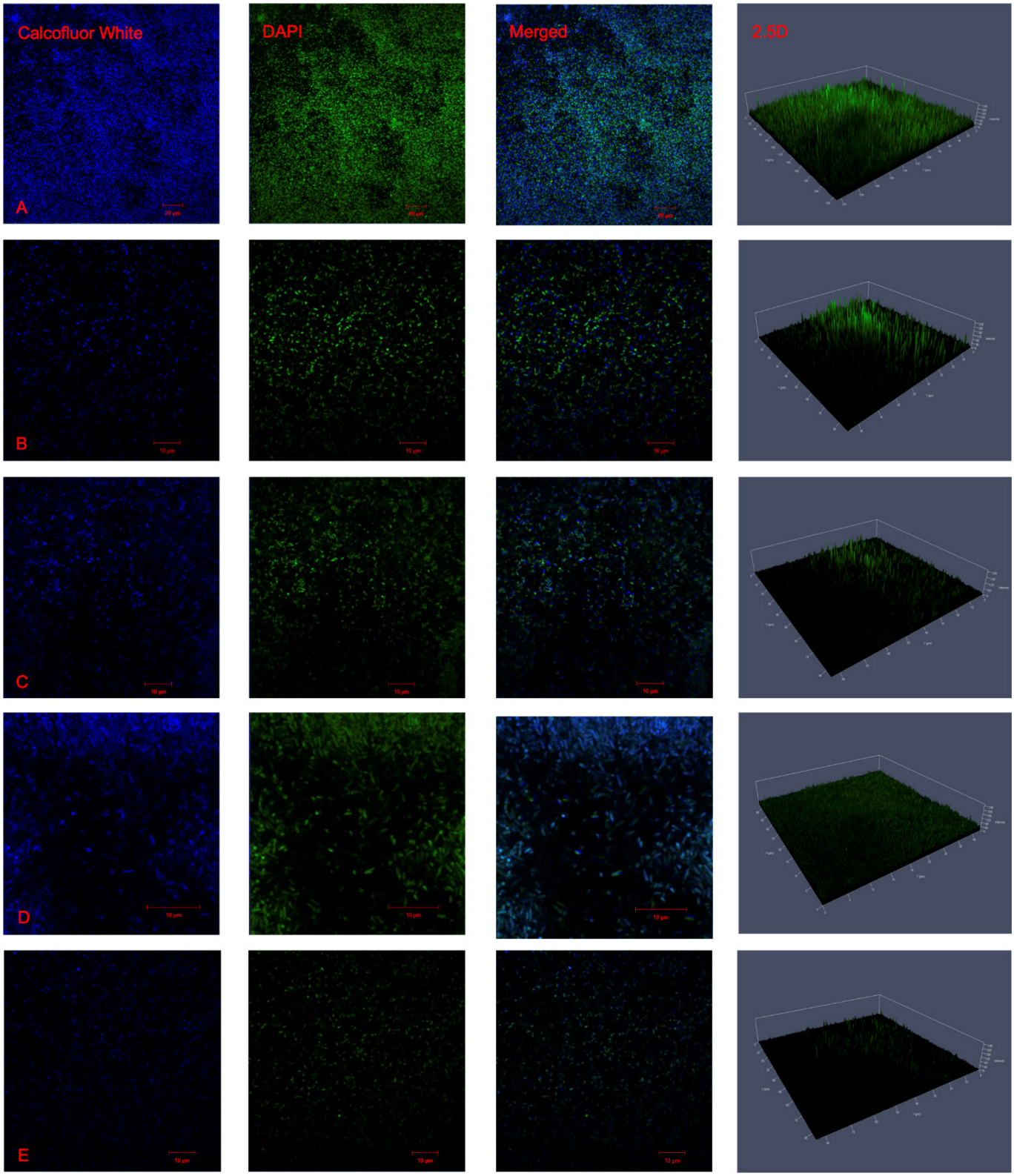
CLSM imaging was done to further confirm the results of the microtiter plate assay with visible effects on the biofilm's structure. The efficacy of our treatments on reducing the biofilm is shown in (Figure 2). The stains, DAPI (green fluorescence) and Calcofluor White (blue fluorescence) were used for imaging.

Table 3. Microtiter plate assay results.

	Samples	Average	SD	% inhibition	Statistical significance
First plate	NC	0.057167	0.004875	-	
	PC (PAN14)	0.248	0.035031	-	
	PAN14 + 1% ginger	0.2595	0.035258	-	
	PAN14 + 3% ginger	0.241	0.013755	-	
	PAN14 + 5% ginger	0.142	0.026623	42.74%	p = 0.0002, Significant
Second plate	NC	0.063167	0.007387	-	
	PC (PAN14)	0.2515	0.024761	-	
	PAN14 +15% wild blueberry (pulp)	0.247	0.022315	-	
	PAN14 + 20% wild blueberry (pulp)	0.2	0.014112	-	
	PAN14 + 25% wild blueberry (pulp)	0.1595	0.012518	36.50%	p = 0.0001, Significant
Third plate	NC	0.063167	0.007387	-	
	PC (PAN14)	0.2515	0.024761	-	
	PAN14 + 15% wild blueberry (peel and pulp)	0.2555	0.011946	-	
	PAN14 + 20% wild blueberry (peel and pulp)	0.239	0.014199	-	
	PAN14 + 25% wild blueberry (peel and pulp)	0.149167	0.007782	40.68%	p = 0.0001, Significant
Fourth plate	NC	0.058667	0.004457	-	
	PC (PAN14)	0.31	0.072297	-	
	PAN14 + 0.1% PS20	0.2775	0.032983	-	
	PAN14 + 0.2% PS20	0.196667	0.008165	36.50%	p = 00.0034, Significant
	PAN14 + 0.3% PS20	0.1475	0.007287	52.00%	p = 0.0003, Significant
Fifth plate	NC	0.058667	0.004457	-	
	PC (PAN14)	0.31	0.072297	-	
	PAN14 + 0.1% PS80	0.275667	0.046984	-	
	PAN14 + 0.2% PS80	0.198333	0.051918	36.00%	p = 0.0118, Significant
	PAN14 + 0.25% PS80	0.112333	0.017874	63.70%	p = 0.0001, Significant

NC: Negative control; PC: Positive control; PAN14: Pseudomonas aeruginosa PAN14; SD: Standard deviation; -: No significant inhibition/ Inapplicable.

Figure 2. Confocal microscopy of P. aeruginosa (PAN14) biofilms before and after treatment. Images were taken from random fields and repeated twice in separate experiments with same results.



(A) Representative images of the untreated biofilm. (B) Biofilm treated with 5% Ginger. (C) Biofilm treated with 25% wild blueberry pulp. (D) Biofilm treated with 25% wild blueberry peel and pulp. (E) Biofilm treated with 0.25% PS80.

Table 4. Relative expression of exopolysaccharide genes (ndvB, algC and pelC) in P. aeruginosa treated and untreated isolate.

	Relative gene expression			Fold decrease		
Samples	***ndvB***	***pelC***	***algC***	***ndvB***	***pelC***	***algC***
Positive control	1.0000	1.0000	1.0000	-	-	-
Ginger 5%	0.8550	0.3605	0.5203	1.16	2.77	1.92
25% wild blueberry (peel and pulp)	0.0108	0.1273	0.0690	92.59	7.85	14.49
25% wild blueberry (pulp)	0.0027	0.2777	0.1152	363.6	3.6	8.69
0.2% PS20	0.1124	0.0436	0.0143	8.89	22.9	69.9
0.25% PS80	0.0726	0.2481	0.1601	13.77	4	6.2

It was clearly shown that the untreated sample (Figure 2A) and (Figure 1-S) displayed a denser biofilm and a more massive cellular distribution when compared to the treated samples (Figure 2, B-E) and PS20 (data not shown). This was also reflected in the 2.5D image which represents the projection of biofilm's density. The merged stained image revealed that treated biofilms were inhibited in a similar manner, except for PS80- treated sample which had the more dispersed cellular and biofilm distribution.

Gene Transcription Level by RT-qPCR

A reduction in the gene expression level of quorum sensing genes as well as exopolysaccharides genes was observed in *P. aeruginosa* biofilm cells that were treated with 5% ginger extract, 25% wild blueberry extract (peel and pulp/pulp only), 0.2% PS20, and 0.25% PS80 comparatively with the control (PAN14). The exopolysaccharides and QS-inducible genes were down expressed in a fold decrease that ranges from 1.16 to 363.6 and 1.34 to 10.4 respectively (Table 4). As for QS genes, our results have shown that the reduction of genes expression in RhlI/ RhlR system was higher than that of the LasI/LasR system genes (Table 5).

The highest reduction in *pelC* gene expression was 22.9 folds, comparatively with the positive control, upon treating with 0.2% PS20. On the other side, *ndvB* and *algC* genes expression were sharply decreased by 363.6 and 69.9 folds when biofilm cells were treated with 25% blueberry pulp extract and 0.2% PS20 respectively.

Furthermore, quorum sensing genes' relative expression was reduced with all treatments, although the reduction wasn't as high as that of EPS genes and the highest reduction recorded was mainly in *rhlR* gene expression with a 10.4 folds' decrease upon treating with 0.2% PS20. Moreover, the reliability of results was assured using the BioRad CFX manager software by the one-peak melting curves obtained for each RT-qPCR procedure.

Discussion

The ability to affect biofilm formation held by the diverse array of chemical structures harbored by natural products opens the door for testing the latter for antibiofilms [24]. These structures constitute more than 40% of the antiinfective drugs [37]. However, using the already discovered antibiofilms is still problematic as not much is known about them [38]. Although previous studies have shown the importance of ginger and polysorbates in combating bacterial biofilms, and proved the anti-quorum sensing activity of wild blueberry [32], none of them studied the molecular mechanism through which biofilm inhibition occurs.

This study demonstrates that ginger, wild blueberry, and polysorbates 20 and 80 were able to reduce biofilm formation in a concentration dependent manner (Table 3) without affecting the growth of *P. aeruginosa*, except for 10% ginger which was shown to delay the growth (Figure 1). The results of the microtiter plate were consistent with the CLSM biofilm imaging results (Figure 2). Green fluorescence, which indicates the presence of viable cells and/or eDNA in biofilm matrix, was found to be more concentrated in the positive control (untreated) than that of treated samples. The later showed dispersed cells and lower biofilm density,

Table 5. Relative expression of quorum sensing genes (lasI, lasR, rhlI, and rhlR) in P. aeruginosa treated and untreated isolate.

	Relative gene expression				Fold decrease			
Samples	***lasI***	***lasR***	***rlhI***	***rlhR***	***lasI***	***lasR***	***rhlI***	***rhlR***
Positive control	1.0000	1.0000	1.0000	1.0000	-	-	-	-
5% Ginger	0.7424	0.717	0.3745	0.1579	1.34	1.39	2.67	6.3
25% wild blueberry peel and pulp	0.5454	0.4935	0.2339	0.2059	1.83	2	4.27	4.85
25% wild blueberry pulp	0.5674	0.538	0.4219	0.2329	1.76	1.85	2.36	4.29
0.2% PS20	0.5948	0.5066	0.2806	0.0905	1.68	1.97	3.56	10.4
0.25% PS80	0.51	0.4868	0.3152	0.3111	1.96	2.05	3.17	3.21

as revealed by the Calcofluor white stain, suggesting that 5% ginger, 25% blueberry peel and pulp, 25% blueberry pulp, 0.2% PS20, and 0.25% PS80 were effective at inhibiting biofilm formation in *P. aeruginosa* (PAN14).

At the molecular level, the RT-qPCR has shown a reduction in the gene expression of both EPS and QS genes with a higher reduction in the RhlI/RhlR system than that of the LasI/LasR, as indicated by (Table 4) and (Table 5) respectively. The fact that *pelC* and *ndvB* genes encode for glycosyltransferases, and *algC* encodes for a phosphomannomutase/phosphoglucomutase (PMM/PGM), which are all required for biofilm synthesis and development [10]. In addition, *lasI* /*rhlI* and *lasR*/*rhlR* genes encode for enzymes and receptors that produce and interact with acyl homoserine lactones (AHLs), which are essential in cell to cell attachments [38]. Our treatments showed that their biofilm inhibitory effect is governed by many genes and various cellular cascades.

Nonetheless, it's known that phenols as well as polyphoenols represent a large class of plants' metabolites [39]. The fact that wild blueberry and ginger have phenols as their main constituents [40,41] and since these are able to bind to receptors and enzymes through their alkyl chains [20,42], it's presumed that the alkyl chains in ginger and wild blueberry extracts, were able to bind to the studied enzymes and block their function in synthesizing exopolysaccharides and acylhomoserine lactones. Furthermore, as for the quorum sensing system, the alkyl chains can share a similarity with AHLs' structures [20] and this allowed them to bind to AHLs' receptors (LasR, RhlR), thus preventing their binding to these receptors. However, these hypotheses require further examination.

Interestingly, the analysis has shown that the effect of the treatments used on RhlI/RhlR system was greater than that on LasI/LasR system (Table 5); this means that the extract's effect on RhlI and RhlR was more favoured.

Moreover, this study is the first to show the effect of polysorbates (PS20, PS80) on *Pseudomonas aeruginosa* biofilm-forming genes. The inhibitory effect of polysorbates exerted on bacterial biofilms has been always thought to be physical [29], however our results demonstrated that polysorbates are interfering with the biofilm molecular cascades in a way that reduces the expression of biofilm-associated gene, thus inhibiting its formation.

Conclusion

In conclusion, these results form a promising approach to help eradicating biofilms in healthcare facilities. Further studies are needed to investigate the exact mechanism of binding between extracts' compounds and polysorbates to the receptors/enzymes implicated in biofilm formation. Also, future studies are required for assessing the activity of the treatments used in *in-vivo* mouse models. Clinically, the applicability of this study can be through the passivation of antibiofilm molecules on indwelling medical devices, or using these molecules in pharmaceutical research for formulating antibiofilm drugs.

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Annex – Supplementary Items

Supplementary Figure 1. CLSM image of P. aeruginosa untreated biofilm.