Modulation of *Pseudomonas aeruginosa* Biofilm Architecture Using Natural Carotenoids: An Innovative Approach to Enhance Antibiotic Efficacy

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INTRODUCTION

Antibiotic resistance is a growing global concern, with the World Health Organization ranking Pseudomonas aeruginosaamong into the high-priority pathogens[1]. This Gram-negative opportunistic bacterium is a major cause of hospital-acquired infections, largely due to its ability to form biofilms[4]. Carotenoids have emerged as promising candidates for disrupting biofilm architecture, thereby potentially reducing the virulence and persistence of *P. aeruginosa*[2,3]. In this context, our research focuses on evaluating the potential of pure carotenoids, such as β-carotene and astaxanthin, and on exploring the diverse natural synergies of carotenoids provided by biological sources.

METHODS

The **MIC/B test** identifies the lowest antimicrobial concentration that inhibits growth (MIC) and kills ≥99.9% of bacteria (MBC). Turbidity reveals the MIC, while subculturing confirms the MBC, distinguishing bacteriostatic from bactericidal effects.

Bacterial growth monitoring is performed by measuring the optical density (OD_{600}) of cultures every 30 minutes over 24 hours thanks to BioTek SYNERGY H1 microplate reader.

Pyocyanin production. Pyocyanin is quantified from culture supernatants by chloroform extraction followed by acid back-extraction; the acidified phase's absorbance is read at 520 nm and normalized to culture density (OD_{600}).

The **crystal violet (0,1%) assay** quantifies biofilm formation by staining surface-attached cells with crystal violet dye. After washing and solubilization, absorbance measurement reflects biofilm biomass.

Bacterial motility is evaluated through **swimming, swarming**, and **twitching** assays, each are performed on media with specific agar concentrations: swimming in soft agar (0.3%) within the liquid–agar matrix, swarming across the surface of semi-solid agar (0.5%), and twitching at the agar–plastic interface of solid medium. Motility capacity is assessed by measuring the diameter of the bacterial halos formed.

Scanning Electron Microscopy (SEM). SEM (JEOL JSM- 7200F) provides high-resolution images of bacterial biofilms by scanning metal-coated (Pt/Au- 60:40) samples with an electron beam.

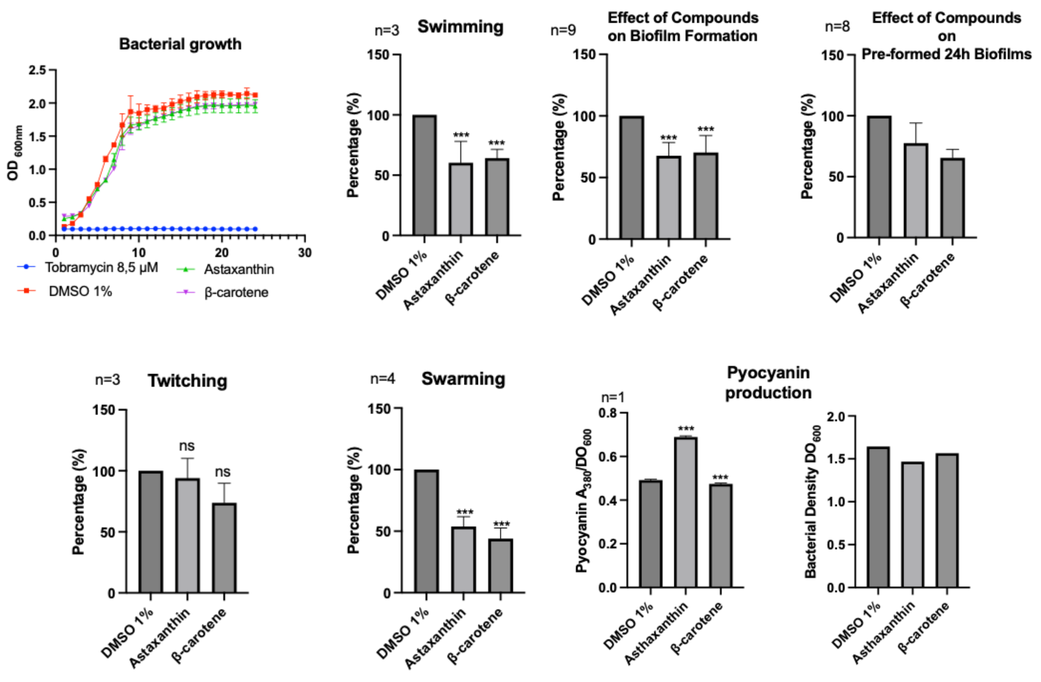
Digital Optical Microscopy. Digital light microscopy (HIROX HRX 01)enables direct observation of 48h biofilms under hydrated conditions, capturing global morphology and coverage on abiotic surfaces.

Extraction procedure. Depending on their water content, fruits were used fresh, oven-dried, or lyophilized. The pulp was extracted several times with acetone until no more color appeared, and all extracts were combined. A second extraction was then carried out with petroleum ether (60–40), and the solvent was finally evaporated

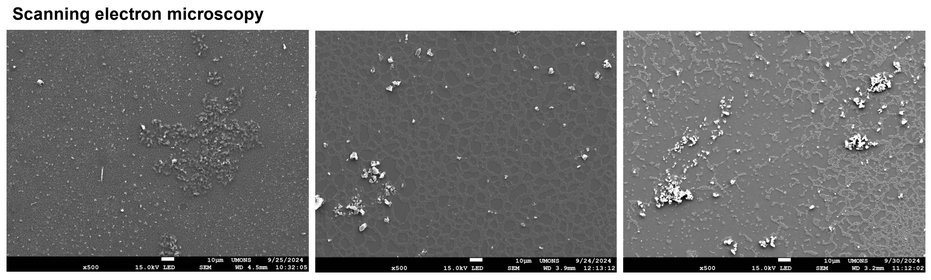
Carotenoid-rich extracts were analyzed by **HPLC–MS** using an Agilent Technologies 1260 Infinity II DAD detector (253nm). Separation was performed on a Phenomenex YMC Carotenoid C30 column (150 × 4.6 mm, 3 µm) with two mobile phases: methanol/water (95:5, v:v) and methanol/methyl tert-butyl ether (30:70, v:v).

RESULTS

Astaxanthin & β-carotene (200µM)

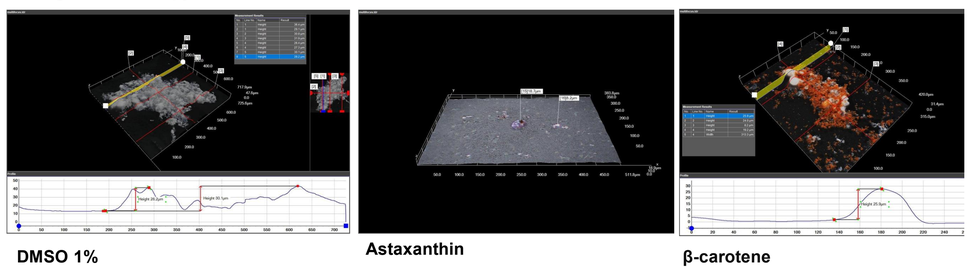


Scanning electron microscopy



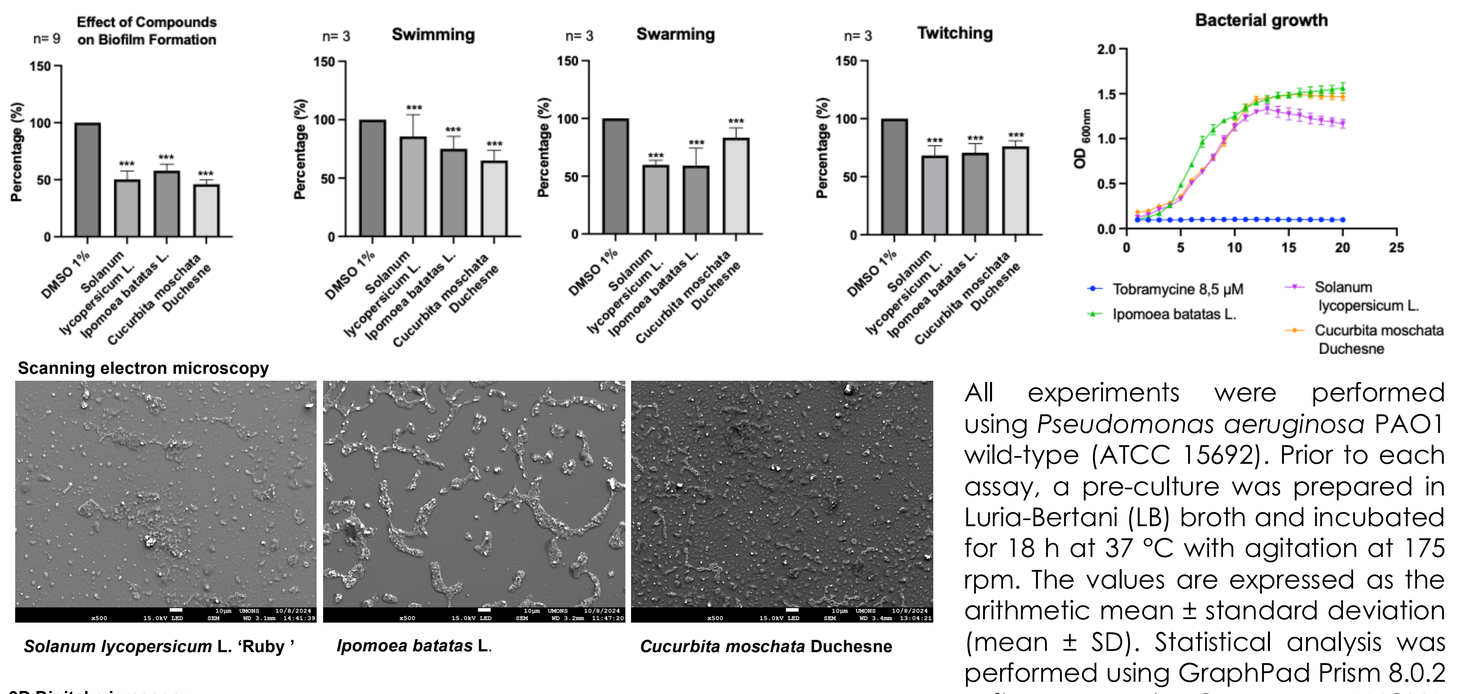
DMSO 1% Astaxanthin β-carotene

3D Digital microscopy



DMSO 1% Astaxanthin β-carotene

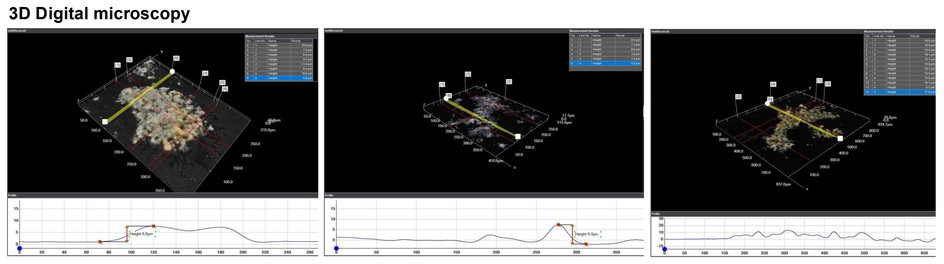
Carotenoid-Rich Plant Extracts (100 µg/mL)



Scanning electron microscopy

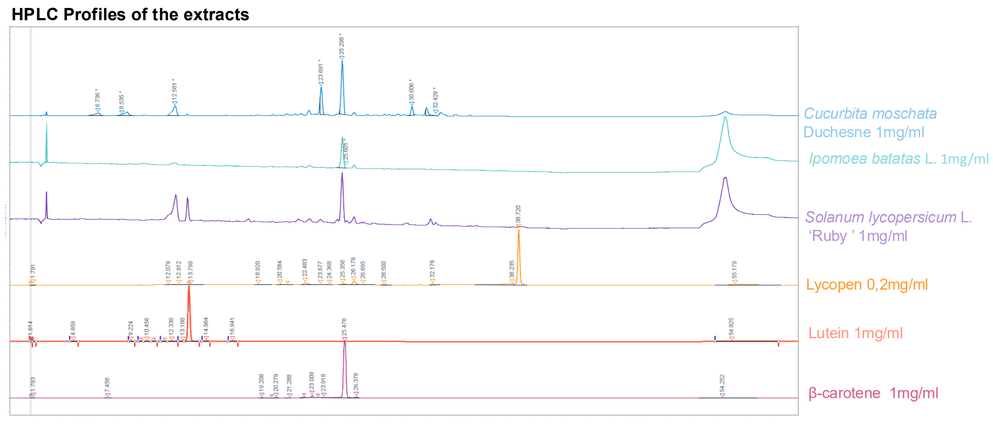
Solanum lycopersicum L. 'Ruby ' *Ipomoea batatas* L. *Cucurbita moschata* Duchesne

3D Digital microscopy

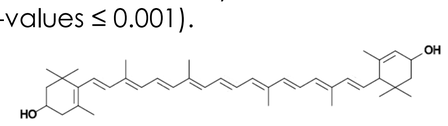


Solanum lycopersicum L. 'Ruby ' *Ipomoea batatas* L. *Cucurbita moschata* Duchesne

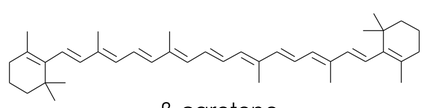
HPLC Profiles of the extracts



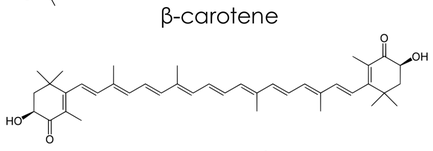
All experiments were performed using *Pseudomonas aeruginosa* PAO1 wild-type (ATCC 15692). Prior to each assay, a pre-culture was prepared in Luria-Bertani (LB) broth and incubated for 18 h at 37 °C with agitation at 175 rpm. The values are expressed as the arithmetic mean ± standard deviation (mean ± SD). Statistical analysis was performed using GraphPad Prism 8.0.2 software and One-way ANOVA followed by Tukey's multiple comparison test. Asterisks indicate samples that are significantly different from DMSO, dimethyl sulfoxide (P-values ≤ 0.001).



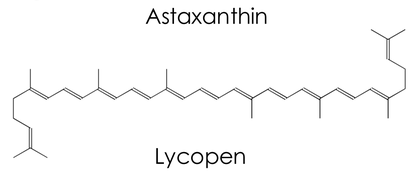
Lutein



β-carotene



Astaxanthin



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DISCUSSION

Astaxanthin and β-carotene neither altered the growth of *Pseudomonas aeruginosa* nor displayed a minimum inhibitory concentration (MIC) within the tested range, supporting an **antivirulence rather than antimicrobial** mode of action. Astaxanthin primarily perturbed flagellum-dependent motilities (**swimming, swarming**), had little effect on twitching, and paradoxically increased pyocyanin—unlike lutein/zeaxanthin, which typically reduce pyocyanin via quorum-sensing (QS) inhibition and limit early biofilm formation[3]. This pattern could point to compound-induced membrane stress that activates QS-regulated pyocyanin biosynthesis; such membrane perturbation might also alter membrane permeability and thereby the diffusion/partitioning of acyl-homoserine lactones, the QS autoinducers[6]. However, these latter observations require replication and should be interpreted with caution. **β-carotene more strongly reduced swarming**; while less active on nascent biofilms, it more effectively destabilized 24-h biofilms. Among plant extracts, ***Cucurbita* showed the most pronounced antibiofilm effect (>50%)** and the strongest inhibition of swimming, whereas ***Solanum* and *Ipomoea* more markedly impaired swarming**; twitching was generally the least affected phenotype. These profiles are consistent with structure–function relationships: xanthophylls (lutein, zeaxanthin) can target QS circuitry[2,3], whereas carotenes (β-carotene, lycopene) more often perturb membrane/matrix-dependent motility and biofilm traits[5]. Microscopy further suggests interactions with the biofilm matrix, in line with the literature. The data may also point to c-di-GMP–mediated switching and, for swarming, possible effects on the Rhl/rhamnolipid pathway—supporting targeted exploration of naturally occurring carotenoids and carotenoid-rich extracts as promising antivirulence scaffolds.

TAKE HOME MESSAGE

Antivirulence rather than antimicrobial: neither astaxanthin, β-carotene or plant extracts affects growth.
Astaxanthin: inhibits flagellum-driven motilities (swimming, swarming).
β-carotene: inhibits flagellum-driven motilities (swimming, swarming); less active on nascent biofilms but destabilizes 24-h pre-formed biofilms.
Plant extracts: *Cucurbita* = best antibiofilm activity (>50%) and strongest inhibition of swimming; *Solanum* and *Ipomoea* mainly impair swarming; twitching is overall the least affected; microscopy suggests interaction with the biofilm matrix.

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