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Spondias tuberosa Arruda (Anacardiaceae) ethanolic leaf extract as a source of natural products inhibiting quorum sensing and pyocyanin production in planktonic *Pseudomonas aeruginosa*

A.S. Tareau [a,b], P.E. Bodet [c], J.R. Guedes da Silva Almeida [d], M. Barreau [a,b], O. Lesouhaitier [a,b], A. Tahrioui [a,b], R. Gonçalves de Oliveira Junior [e], R. Grougnet [e], P. Sitarek [f], L. Quintans-Junior [g], K. Baranger [h], S. Chevalier [a,b], L. Picot [h,*]

[a] CBSA UR 4312, Univ Rouen Normandie, Université Caen Normandie, Normandie Univ, Rouen F-76000, France
[b] Plateforme de Sécurité Sanitaire Ébroïcienne (PS2E), Univ Rouen Normandie, Normandie Univ, CBSA UR 4312, Rouen F- 76000, France
[c] UMR CNRS 7266 LIENSs, Platform for the High Resolution Analysis of Biomolecules, La Rochelle Université, La Rochelle 17042, France
[d] NEPLAME, Universidade Federal do Vale do São Francisco, Petrolina 56304-205, Brazil
[e] UMR CNRS 8038 CiTCoM, Université Paris Cité, Paris 75006, France
[f] Department of Medical Biology, Medical University of Lodz, Lodz, Poland
[g] LANEF, Universidade Federal de Sergipe, Aracaju 49100-000, Brazil
[h] UMR CNRS 7266 LIENSs, La Rochelle Université, La Rochelle 17042, France

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ABSTRACT

Pseudomonas aeruginosa is a multidrug-resistant pathogen whose virulence is tightly regulated by quorum sensing (QS). Targeting QS offers a promising anti-virulence strategy by disarming pathogens without promoting resistance. Here, we screened Brazilian medicinal plant extracts for QS inhibition and identified the ethanolic leaf extract of *Spondias tuberosa* Arruda (STF) as a potent suppressor of pyocyanin production in planktonic *P. aeruginosa* cultures, without affecting bacterial growth. STF disrupted QS signaling, as demonstrated by the decreased production of QS mediators detected using a biosensor strain. Phytochemical profiling revealed candidate compounds potentially responsible for this activity. These findings highlight *S. tuberosa* as a promising natural source of QS inhibitors capable of attenuating *P. aeruginosa* virulence, particularly during the early stages of infection, and open perspectives for future research into its potential interest in anti-virulence therapeutic approaches.

1. Introduction

Pseudomonas aeruginosa is a Gram-negative, opportunistic pathogen responsible for a wide range of infections, particularly in immunocompromised individuals (Hernández-Jiménez et al., 2022; Lyczak et al., 2000; Moradali et al., 2017). Its virulence is tightly regulated by a complex QS system that orchestrates the expression of numerous pathogenic determinants (Lee and Zhang, 2015; Nadal Jimenez et al., 2012). The QS network in *P. aeruginosa* comprises three interrelated signaling systems, Las, Rhl, and PQS, which govern the coordinated production of key virulence factors such as pyocyanin, elastase, and rhamnolipids (Lee and Zhang, 2015; Williams and Cámara, 2009). Pyocyanin, a redox-active phenazine pigment, promotes the generation of reactive oxygen species (ROS), resulting in oxidative tissue damage and suppression of host immune responses (Lau et al., 2004). Elastase contributes to tissue invasion by degrading structural components of the extracellular matrix, while rhamnolipids facilitate biofilm development and the lysis of host cells (Bever and Iglewski, 1988). Together, these virulence factors enhance the persistence and resilience of *P. aeruginosa*, positioning it as a critical member of the ESKAPE group—an acronym

* Correspondence to: UMR CNRS 7266 LIENSs, Institut du Littoral et de l'Environnement, 2 rue Olympe de Gouges, La Rochelle 17000, France.
E-mail addresses: anne-sophie.tareau@univ-rouen.fr (A.S. Tareau), pierreedouard.bodet@univ-lr.fr (P.E. Bodet), jackson.guedes@univasf.edu.br (J.R. Guedes da Silva Almeida), magalie.barreau@univ-rouen.fr (M. Barreau), olivier.lesouhaitier@univ-rouen.fr (O. Lesouhaitier), ali.tahrioui@univ-rouen.fr (A. Tahrioui), raimundo.goncalves-de-oliveira-junior@parisdescartes.fr (R. Gonçalves de Oliveira Junior), raphael.grougnet@parisdescartes.fr (R. Grougnet), przemyslaw.sitarek@umed.lodz.pl (P. Sitarek), lucindo@academico.ufs.br (L. Quintans-Junior), kevin.baranger@univ-lr.fr (K. Baranger), sylvie.chevalier@univ-rouen.fr (S. Chevalier), laurent.picot@univ-lr.fr (L. Picot).

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denoting highly virulent, antibiotic-resistant microorganisms responsible for severe hospital-associated infections (Boucher et al., 2009; Pendleton et al., 2013). Considering the growing prevalence of multidrug-resistant *P. aeruginosa*, there is an urgent demand for novel therapeutic approaches. A promising alternative lies in targeting bacterial virulence instead of viability, thereby reducing selective pressure and the emergence of resistance. QS inhibition interrupts the regulation of virulence gene expression, offering a strategic means to attenuate pathogenicity (Defoirdt, 2018; Kalia, 2013; LaSarre and Federle, 2013; Rasko and Sperandio, 2010). In this context, natural products derived from medicinal plants represent a valuable reservoir of potential QS inhibitors, that can include various compounds such as alkaloids, polyphenols, terpenoids, and organosulfur compounds. These bioactive molecules have already shown the ability to disrupt QS networks and decrease the production of virulence determinants such as pyocyanin, elastase, and rhamnolipids (Choo et al., 2006; Jakobsen et al., 2012; Tahrioui et al., 2020; Vadakkan et al., 2024). Brazil, a country renowned for its vast biodiversity, contains an extraordinary array of medicinal plants with demonstrated antimicrobial potential, many of which have long been used in traditional medicine for the prevention and treatment of infections (Almeida et al., 2012, 2006; de Lima-Saraiva et al., 2017; Nascimento et al., 2000). This unique ecological biodiversity presents an ideal opportunity for the discovery of novel QS inhibitors capable of disrupting bacterial communication without triggering resistance mechanisms. Identifying such compounds from Brazilian flora could drive opportunities for innovative anti-infective strategies, particularly against multidrug-resistant pathogens like *P. aeruginosa*, and contribute to global efforts to combat antimicrobial resistance. Several plant species from the Myrtaceae, Fabaceae, and Asteraceae families have already exhibited activity against *P. aeruginosa*, primarily through mechanisms such as QS interference, membrane destabilization, and efflux pump inhibition (D'Aquila et al., 2023; Fabri et al., 2011; Masuku et al., 2023; Musthafa et al., 2017; Rosado-Vallado et al., 2000; Tahrioui et al., 2020). In this study, a screening of 20 extracts obtained from 10 Brazilian medicinal plants was conducted to identify candidates capable of suppressing *P. aeruginosa* virulence. Among the extracts tested, the ethanolic leaf extract of *Spondias tuberosa* Arruda (STF) stood out for its significant inhibition of pyocyanin production in planktonic cells. STF markedly decreased pyocyanin levels without impairing bacterial growth, suggesting a mechanism of action based on QS disruption rather than bactericidal activity. Subsequent bioassays confirmed a decrease in the production of QS signaling molecules, supporting the hypothesis of interference with QS-regulated pathways. Phytochemical analysis of STF revealed several candidate compounds potentially responsible for this anti-virulence effect. These findings position *Spondias tuberosa* leaf ethanolic extract as a promising source of natural QS inhibitors and support further investigations to valorize its bioactive compounds for antimicrobial applications.

2. Materials and methods

2.1. Plant material collection and extract preparation

All plants studied were collected in areas of the Caatinga biome (semiarid vegetation, Brazil) and the botanical identification was made by comparison with voucher specimens deposited at the Herbário Vale do São Francisco (HVASF). All necessary permissions for the collection and use of plant material were obtained. The study was conducted under SisGen authorization (Register #A654697) in accordance with Brazilian biodiversity regulations (Law No. 13.123/2015). *Spondias tuberosa* was harvested on April 2024 at Sítio Asa Branca in Petrolina, state of Pernambuco, Brazil (Coordinates: S 9°30'86.54", W 40°72'07.94''). The Caatinga biome is a semi-arid and unique North-eastern Brazilian ecosystem characterized by high endemism and a rich diversity of medicinal plants traditionally used by local communities for managing infections and inflammatory conditions. The plant material was washed with water, dried at a temperature of 45 °C for 72 h, and then, pulverized and exhaustively macerated with solvents at room temperature with solvent renewal every 72 h (three repetitions) as detailed in Table 1. Ethanol and methanol were selected to prioritize the extraction of polar compounds, particularly polyphenols and flavonoids, known for their quorum sensing inhibitory potential. The choice of plant part was based on traditional uses and availability; for instance, stem barks were selected for *Hymenaea martiana* and *Piptadenia viridiflora* due to their recognized medicinal value, while leaves were prioritized for Spondias tuberosa and other species because of their metabolic richness and easier renewability. The extractive solutions were concentrated under low pressure using a rotary evaporator (Quimis, Brazil) to obtain the crude extracts. For some extracts, further fractionation was performed using a secondary solvent as detailed in

Table 1**. Summary of plant extracts and fractions tested in this study.** All plant materials were collected in April 2024 from Sítio Asa Branca (Petrolina, Pernambuco, Brazil), within the Caatinga biome. Extracts were prepared by exhaustive maceration of dried and pulverized plant material using appropriate solvents, followed by concentration under reduced pressure. The initial weights of the dried plant materials ranged from 10 to 500 g, depending on the species and the plant part used. Some extracts were further fractionated as indicated. The table details the extract code, corresponding plant species, botanical family, voucher specimen number, type of extract or fraction, physical appearance and final extract weight.

2.2. Bacterial strains and culture conditions

The *P. aeruginosa* H103, a prototroph of the wild-type strain PAO1, was provided by Prof. Robert E W Hancock from the University of British Columbia (Hancock and Carey, 1979). The PAO1 mini-CTX pqsA::lux biosensor strain was provided by Prof. Miguel Camara from the University of Nottingham (Fletcher et al., 2007). To obtain it, single-copy luxCDABE transcriptional fusions of the pqsA, pvdS, and pvdE promoters were integrated into the chromosome of the PAO1 pqsA mutant using the mini-CTXlux system. (Becher and Schweizer, 2000; Diggle et al., 2007). A 532 bp fragment containing the pqsA promoter region was amplified and cloned into pBluescript KS+ digested with *Hin*dIII and *Bam*HI. The fragment was excised with *Hin*dIII and *Bam*HI and cloned into similarly digested mini-CTX1ux resulting in the mini-CTX pqsA::lux biosensor strain (Becher and Schweizer, 2000). *P. aeruginosa* H103 was grown in Luria-Bertani broth containing 171 mM (10 $g.L^{-1}$) NaCl (LB, BD Difco ™, Fisher Scientific, Illkirch-Graffenstaden, France), for 18 h at 37 °C under shaking (180 rpm). All experiments in this study were conducted using planktonic cultures of *P. aeruginosa* H103. This model is widely recognized as representative of the bacterial behavior during acute infection phases, where *P. aeruginosa* predominantly exists in a free-living, planktonic state prior to biofilm establishment. This approach ensures that the observed effects of plant extracts on quorum sensing, pyocyanin production, and 4-hydroxy-2-alkylquinolines (HAQ) signaling are relevant to the early stages of infection, where therapeutic interventions targeting virulence factors could be most effective.

2.3. Pyocyanin quantification

P. aeruginosa H103 exposed to plant extracts at serial concentrations ranging from 8 to 256 $\mu g \cdot mL^{-1}$, following standard practices used in antibiogram assays, or DMSO 1 % (control) was grown in LB in a 96-well microtiter plate (Thermo Fisher Scientific, Nunc™, Villebon-sur-Yvette, France) at 37 °C for 24 h with shaking (180 rpm). After incubation for 24 h, $A_{580\ nm}$ was measured to determine the bacterial growth. The pyocyanin quantification assay was carried out as previously described (Azuama et al., 2020; Tahrioui et al., 2020) and the 64 $\mu g \cdot mL^{-1}$ was determined as a discriminant concentration among plant extracts. For pyocyanin quantification, one volume of chloroform was added to one volume of culture supernatants. After centrifugation, the chloroform

Table 1
Fractionation was performed on selected extracts to improve compound identification and comparative analysis of inactive versus active extracts. Although STF showed the highest activity, fractionation of less active extracts was conducted to explore chemical diversity and verify whether similar compounds were present.

Extract code	Species name	Botanical Family	Nature of the extract	Voucher specimen number	Weight (g)
BC	*Bauhinia cheilantha*	*Fabaceae*	Ethanolic extract of the leaves	2241	88.0
EDSAS	*Annona spinescens*	*Annonaceae*	Dichloromethane extract of the seeds	25507	55.0
EHFAS			Hexanic extract of the leaves		17.0
EHSAS			Hexanic extract of the seeds		15.0
EMCAS			Methanolic extract of the stembarks		33.0
EMFAS			Methanolic extract of the leaves		35.0
EMSAS			Methanolic extract of the seeds		33.0
EMTAS			Methanolic extract of the twigs		33.0
ES	*Encholirium spectabile*	*Bromeliaceae*	Ethanolic extract of the leaves	6443	10.0
HM	*Hymenaea martiana*	*Fabaceae*	Ethanolic extract of the stembarks	6444	10.0
MN	*Morus nigra*	*Moraceae*	Ethanolic extract of the leaves	1764	322.0
MT	*Mimosa tenuiflora*	*Fabaceae*	Ethanolic extract of the stembarks	16714	29.0
PC	*Passiflora cincinnata*	*Passifloraceae*	Ethanolic extract of the leaves	22870	12.0
FDPC			Dichloromethane fraction of PC		2.9
FEPPC			Petroleum ether fraction of PC		0.5
FMPC			Methanolic fraction of PC		5.7
PF	*Passiflora foetida*	*Passifloraceae*	Ethanolic extract of the leaves	2413	28.7
PV	*Piptadenia viridiflora*	*Fabaceae*	Ethanolic extract of the stembarks	28	105.0
STC	*Spondias tuberosa*	*Anacardiaceae*	Ethanolic extract of the stembarks	18319	483.0
STF			Ethanolic extract of the leaves		252.0

blue layer was collected and ½ volume of 0.5 M HCl was added. After a second centrifugation, the red pink HCl layer was collected and the $A_{520\ nm}$ was recorded (Spark 20 M multimode Microplate Reader, Tecan Group Ltd., Crailsheim, Germany). The data were normalized to the bacterial cell density ($A_{520\ nm}/A_{580\ nm}$) and expressed as a percentage of the control value. This pyocyanin inhibition assay enabled the identification of the most active extract, which was subsequently selected for further experiments.

2.4. Evaluation of Spondias tuberosa Arruda ethanolic leaf extract (STF) on Pseudomonas aeruginosa H103 growth kinetics

To assess the effect of STF on *P. aeruginosa* H103 growth kinetics, bacterial cultures were grown in the presence or absence of STF at 64 $\mu g{\cdot}mL^{-1}$. An absorbance measure at 580 nm was performed every 30 min using the Spark 20 M multimode Microplate Reader controlled by Spark Control™ software Version 2.1 (Tecan Group Ltd., Crailsheim, Germany). The data were plotted, and each point indicates the mean ± standard error of the mean (SEM) of $A_{580\ nm}$ values. Each set of experiments was performed at least three times.

2.5. Extraction and quantification of 4-hydroxy-2-alkylquinolines (HAQs)

Measuring the production of HAQ molecules—key quorum sensing mediators in *P.aeruginosa* such as PQS, HHQ, and HQNO—provides a relevant indicator of quorum sensing activity, thereby enabling the evaluation of the STF extract's impact on this regulatory network. Planktonic cultures of *P. aeruginosa* H103, either untreated (dimethyl sulfoxide (DMSO) 1 % control) or treated with 64 $\mu g{\cdot}mL^{-1}$ of STF, were incubated for 24 h at 37 °C with shaking (180 rpm) and then subjected to HAQ molecule extraction following the protocol previously described by Tahrioui et al. (Tahrioui et al., 2019). The quantification of HAQs was performed using a combined spectrophotometric and luminometric microplate assay with the biosensor strain PAO1 *pqsA CTX-lux::pqsA* (Diggle et al., 2011). After overnight culture, the biosensor strain was adjusted with fresh LB medium to an optical density (OD_{580nm}) of 1. For the assay, 5 µL of crude HAQ extracts were diluted in 100 µL of LB and mixed with 100 µL of a 1:50 dilution of the biosensor strain. Bioluminescence and OD_{580nm} were recorded every 15 min over 24 h at 37 °C using a Spark 20 M multimode microplate reader, managed by SparkControl™ software Version 2.1 (Tecan Group Ltd.). Bioluminescence values, expressed as relative light units (R.L.U), were normalized to the $OD_{580\ nm}$ of the culture suspensions.

2.6. Compounds identification by ultra-performance liquid chromatography coupled with diode array detection and high-resolution mass spectrometry (UPLC-DAD-MS/MS)

An Acquity UPLC H-class system (Waters, Milford, CT, USA) coupled to a photodiode array (Waters 2996) and a high-resolution mass spectrometer (a XEVO G2-S Q-Tof equipped with an electrospray ionization source (Waters, Manchester, UK)) were used. The STF extract was solubilized in DMSO at 0.1 $g.L^{-1}$ and diluted tenfold with 5 % acetonitrile (ACN). Prior to injection, the solution was centrifuged at 13,000 rpm for 5 min and filtered through a 0.22 µm polyvinylidene fluoride (PVDF) membrane. The injection volumes were 5 µL for MS analysis and 10 µL for MS/MS and UV detection. Chromatographic separation was performed using a BEH C18 column (2.1 ×50 mm, 1.7 µm particle size, Waters, Manchester, UK) with a mobile phase comprising solvent A (H_2O + 0.001 % formic acid) and solvent B (ACN + 0.001 % formic acid) at a column oven temperature of 25°C. The flow was set at 0.3 mL/min and the gradient for solvents A and B was as follows: 0–3 min, 95 % A and 5 % B; 3–7 min 90 % A and 10 % B; 7–10 min 90 % A and 10 % B; 10–12 min, 85 % A and 15 % B; 10–12 min 85 % A and 15 % B; 12–14 min, 85 % A and 15 % B; 14–16 min, 80 % A and 20 % B; 16–19 min, 80 % A and 20 % B; 19–21 min, 70 % A and 30 % B; 21–23 min, 70 % A and 30 % B; 23–26 min, 10 % A and 90 % B; 26–29 min, 10 % A and 90 % B; 29–30 min, 95 % A and 5 % B and 30–35 min, 95 % A and 5 % B. The extraction process was carried out using solvents primarily targeting polar compounds, which are particularly relevant because many polar metabolites such as phenolic acids and flavonoid glycosides are known to interfere with quorum sensing in *Pseudomonas* by disrupting AHL-mediated communication, thereby justifying the chromatographic gradient optimized for their early resolution and the limited consideration given to apolar compounds or contaminants eluting at high acetonitrile concentrations. Mass spectrometry data were acquired in both polarities (capillary voltages of +3 kV and −2.5 kV) using an ESI-QTof setup, with *m/z* ranging from 50 to 1200 and scan time of 0.1 s. MSMS analysis were conducted based on an ion intensity threshold of $1000{,}000.s^{-1}$ with collision energies ranging from 10 to 40 V, and a UV analysis was performed in a detection range from 220 to 800 nm and 10 $spectra.s^{-1}$. These parameters provided high-resolution separation and detection, enabling detailed

identification and characterization of compounds in the STF extract. After MS/MS acquisition, the resulting ion spectra were processed using the identification software Sirius4© (Dührkop *et al.*, 2019). Molecular formula determination was first performed in Sirius, allowing the elements C, H, O, N, P, and S, with an MS^2 mass accuracy set to 20 ppm. The 'Predict FPs' module was then applied to infer molecular fingerprints, while 'Search DBs' compared these predicted fingerprints with reference MS/MS data from natural compound databases such as the Human Metabolome Database (HMDB), Chemical Entities of Biological Interest (ChEBI), COlleCtion of Open NatUral producTs (COCONUT), and the Global Natural Products Social Molecular Networking (GNPS) repository, among others. Finally, the 'CANOPUS' module was used to predict compound classes. Molecular formulas were retained when Sirius scores exceeded 75 %, and only compound structures showing a minimum similarity of 60 % were considered for annotation.

2.7. Statistical analysis

Data analysis was performed using GraphPad Prism version 10.2.2. Statistical significance was assessed using Kruskal–Wallis test followed by Dunn's multiple comparaison and Wilcoxon test, as specified in the figure legends.

3. Results

3.1. Reduction of pyocyanin production

Pyocyanin is a blue phenazine pigment and a key virulence factor of *P. aeruginosa*, responsible for the generation and accumulation of reactive oxygen species (ROS), leading to oxidative stress in both prokaryotic and eukaryotic cells (Gonçalves and Vasconcelos, 2021; Lau et al., 2004). This virulence factor enhances *P. aeruginosa*'s competitive advantage over other microorganisms in diverse environments (Gonçalves and Vasconcelos, 2021) and induces apoptosis and senescence in eukaryotic cells (Allen et al., 2005; Managò et al., 2015). Due to its significant role in pathogenicity, pyocyanin is widely recognized as a biomarker of *P. aeruginosa* infections. In this study, we evaluated the effect of several Brazilian plant extracts on pyocyanin production by planktonic cells of *P. aeruginosa*. As shown in Fig. 1, eight extracts significantly reduced pyocyanin levels, with inhibition ranging from 15 % (PC) to 70 % (STF) at 64 μg.mL^{-1}, selected as a discriminating concentration. Notably, STF exhibited the most pronounced effect on pyocyanin production. Based on these findings, STF was selected for further investigation in subsequent assays.

3.2. Effect of the STF extract on Bacterial Growth

The impact of STF on *P. aeruginosa* H103 growth was monitored at 37 °C for 24 h, showing that the leaf extract at 64 μg.mL^{-1} had no impact on planktonic cell growth of H103 strain as compared to the untreated culture (H103 strain grown in presence of 1 % v/v DMSO) (Fig. 2). This result was particularly interesting, as the study aimed to identify extracts that modulate quorum sensing without affecting bacterial viability, thus ensuring that the observed effects on virulence-related phenotypes are not a consequence of impaired growth.

3.3. Effect of the STF extract on Quorum Sensing Modulation

We next sought to assess whether the inhibition of pyocyanin production was directly due to the activity of STF on the Pqs QS system that is known to tightly regulate pyocyanin biosynthesis. *P. aeruginosa* H103 cultures were exposed to STF at 64 μg.mL^{-1} , and then HAQs molecules were extracted twice by ethyl acetate. The production of HAQs was determined using a PAO1 *pqsA* CTX-*pqsA::lux* biosensor strain, which does not produce HAQs molecules but shows proportional quantitative response to exogenous HAQs. The H103 cultures treated with STF showed significant reduced HAQs molecules production, demonstrating that STF exhibited its anti-pyocyanin production effect at least through an anti-QS activity (Fig. 3.).

3.4. Composition of Spondias tuberosa Arruda ethanolic leaf extract and correlation with its anti-virulence activity

The UHPLC-HRMS analysis of the STF extract revealed a complex mixture of 77 compounds across 30 chemical subclasses. Four major chemical families, flavonoids, carbohydrates, carboxylic acids, and amino acid derivatives, accounted for 50.6 % of the extract's composition (Fig. 5., Tables 2. and 3.). Flavonoids, the most abundant class (14.3 %), included glycosides such as rutin ($C_{27}H_{30}O_{16}$), nicotifolin ($C_{27}H_{30}O_{15}$), and cinaroside ($C_{21}H_{20}O_{11}$), as well as *O*-methylated

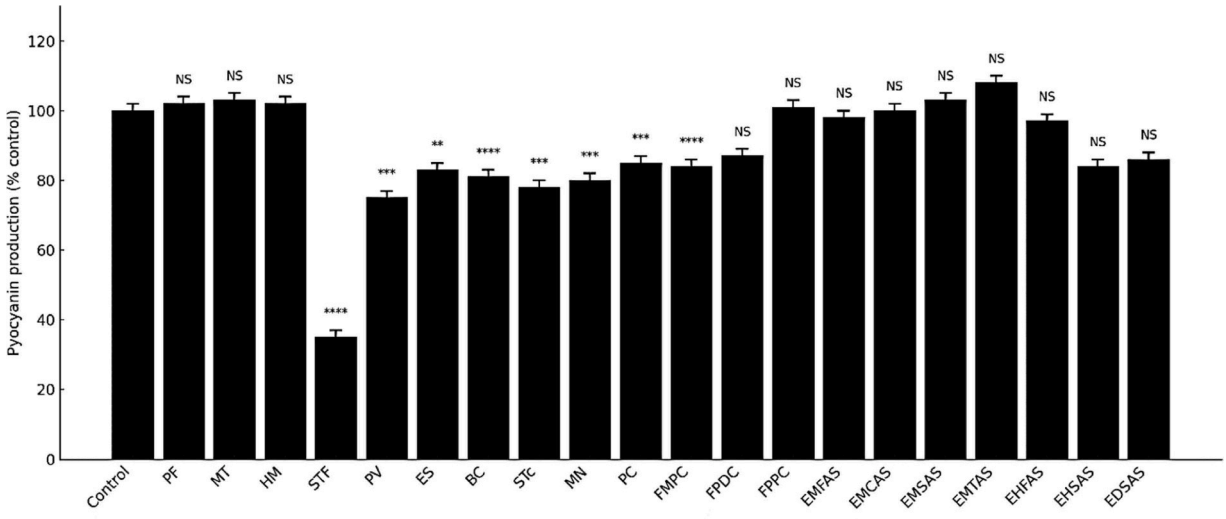


Fig. 1. Inhibitory effect of plant extracts on pyocyanin production in *P. aeruginosa* H103. Plant extracts and fractions were initially tested at concentrations ranging from 8 to 256 μg·mL^{-1} . The figure shows the effect of a selected concentration (64 μg·mL^{-1}), chosen for its discriminating potential, on pyocyanin production by *P. aeruginosa* H103 after 24 h of incubation at 37 °C. Pyocyanin production was quantified spectrophotometrically and expressed as a percentage relative to the untreated control. Statistical significance was determined using Kruskal-Wallis test followed by Dunn's multiple comparison test. NS indicates non-significant differences; **p < 0.01, ***p < 0.001, ****p < 0.0001. Data represent the mean ± SEM of three independent experiments.

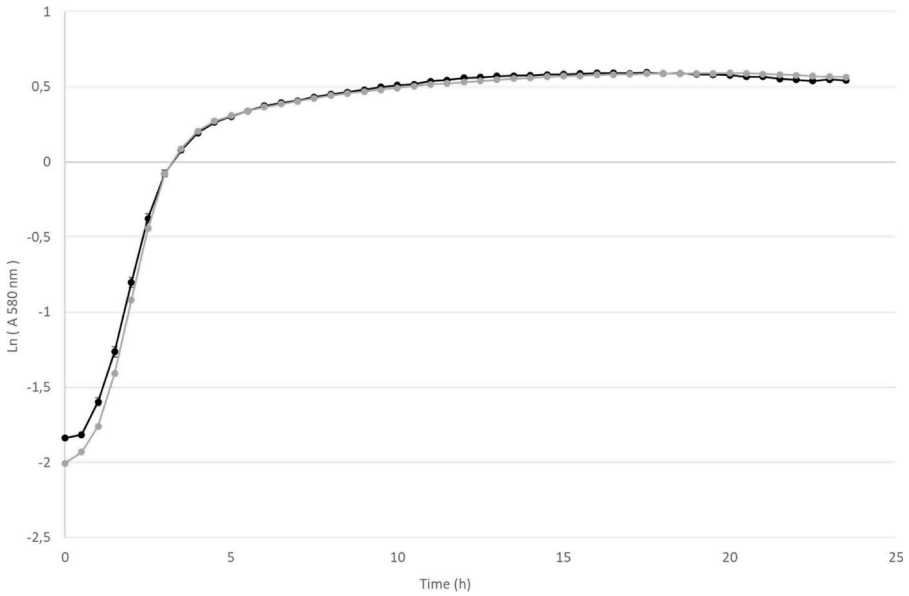


Fig. 2. Effect of STF leaf extract on the planktonic growth of *P. aeruginosa* H103. Growth kinetics of *P. aeruginosa* H103 in LB medium at 37 °C in the presence of 1 % DMSO (H103, black curve) or STF leaf extract at 64 µg·mL^{-1} (grey curve). A_{580nm} was measured every 30 min over 24 h. Data are expressed as the mean ± SEM of the neperian logarithm of absorbance values from four independent experiments. Error bars were very small, indicating a high reproducibility of the assay.

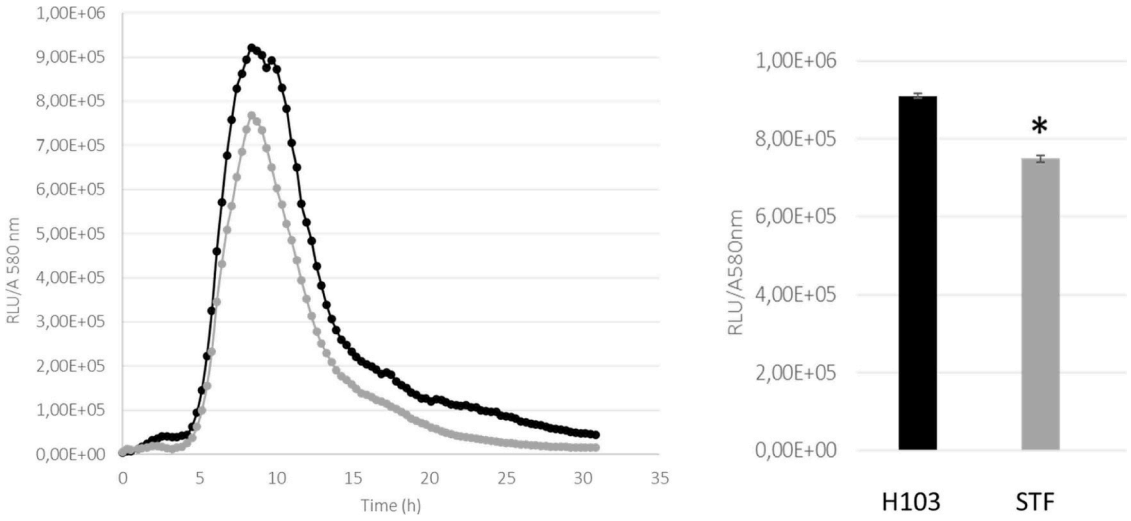


Fig. 3. STF leaf extract inhibits Pqs quorum sensing system activity in *P.aeruginosa* H103. Bioluminescence kinetics (left) and corresponding maximal values (right) of the *P. aeruginosa pqsA::lux* reporter strain exposed to 1 % DMSO (negative control, black curve and bars) or 64 µg.mL^{-1} of the ethanolic leaf extract of *Spondias tuberosa* (STF). Luminescence was monitored over time, and peak values were extracted for comparative analysis. Data represent the mean ± SEM of four independent experiments. Statistical significance was assessed using the Wilcoxon signed-rank test; $p < 0.05$ was considered significant (*).

derivatives such as sakuranetin ($C_{16}H_{14}O_5$) and naringenin dimethyl ether ($C_{17}H_{16}O_5$). Carbohydrates and carbohydrate conjugates (14.3 %) included cerelose ($C_6H_{12}O_6$) and various complex glycosides. Carboxylic acids (13 %) comprised mono-, di-, and tricarboxylic acid derivatives, notably hibiscus acid methyl ester ($C_7H_8O_7$) and hydroxycitric acid dimethyl ester ($C_8H_{12}O_8$). Amino acids, peptides, and analogues represented 9.1 %, including methyl pyroglutamate and ethyl pyroglutamate. Other classes included hydroxycinnamic acids (e.g., ethyl caffeate), fatty acids (e.g., linoleic acid and 10,15-octadecadiynoic acid), benzenediols (e.g., loliolide), glycerophosphocholines (e.g., lysolecithins), and pyridoindoles (e.g., harman-3-carboxylic acid). (Figs. 4, 5 and Tables 2 and 3).

4. Discussion

This study provides compelling evidence that the ethanolic leaf extract of *Spondias tuberosa* Arruda (STF) exhibits strong anti-virulence activity against planktonic *Pseudomonas aeruginosa* by specifically targeting quorum sensing (QS)-regulated pathways controlling pyocyanin production. In particular, STF was shown to significantly inhibit the Pqs system, a key regulator of virulence in *P. aeruginosa* through the production of HAQs molecules such as HHQ and PQS, which control the expression of key factors including pyocyanin (Diggle et al., 2011; Lee and Zhang, 2015). The observed reduction in HAQs was confirmed through bioluminescence assays using a *pqsA::lux* biosensor strain and was not accompanied by any adverse effects on bacterial growth, supporting a non-bactericidal and therefore resistance-limiting mode of action. Phytochemical analysis of STF revealed a chemically diverse

Table 2
UPLC-HRMS data obtained after analysis of the STF extract, listing compounds identified in positive ionization mode. The table includes retention time (RT), mass-to-charge ratio (*m/z*), molecular formula, adduct type, identification score, proposed molecular structure, similarity index, and chemical subclass. The identified metabolites belong to diverse biochemical classes, including carbohydrates, amino acids, flavonoids, fatty acids, and phospholipids, offering insights into the chemical composition and potential bioactivity of the extract.

N°	*m/z*	RT (min)	Mass formula	Adduct	Score	Proposed structure	Similarity	Subclass
2	203.0536	0.44	$C_6H_{12}O_6$	$[M+Na]^+$	100 %	Cerelose	94.55 %	Carbohydrates and carbohydrate conjugates
3	134.1185	0.48	$C_6H_{15}NO_2$	$[M+H]^+$	100 %	2-((2-Methoxyethyl)(methyl)amino)ethanol	81.48 %	Amines
7	300.1446	0.83	$C_{14}H_{21}NO_6$	$[M+H]^+$	96.20 %	(2 R,3S,4 R,5 R,6S)−2-(hydroxymethyl)−6-[[(4-hydroxyphenyl)methylamino]methyl] oxane−3,4,5-triol	73.29 %	Carbohydrates and carbohydrate conjugates
10	144.0664	1.23	$C_6H_9NO_3$	$[M+H]^+$	100 %	Méthyl L-pyroglutamate	98.95 %	Amino acids, peptides and analogues
15	158.0836	3.5	$C_7H_{11}NO_3$	$[M+H]^+$	100 %	Ethyl pyroglutamate	98.10 %	Amino acids, peptides and analogues
17	287.0742	5.04	$C_{10}H_{16}O_8$	$[M+Na]^+$	100 %	[(2 R,3 R,4S,5 R)−5-acetyloxy−2,3,4-trihydroxy−6-oxohexyl] acetate	72.67 %	Carbohydrates and carbohydrate conjugates
27	227.0819	9.05	$C_{13}H_{10}N_2O_2$	$[M+H]^+$	100 %	Harman−3-Carboxylic Acid	90.38 %	Harmana alkaloids
29	197.1174	12.95	$C_{11}H_{16}O_3$	$[M+H]^+$	100 %	Loliolide	67 %	Benzenediols
32	611.1609	14.85	$C_{27}H_{30}O_{16}$	$[M+H]^+$	100 %	Rutine	99.72 %	Flavonoid glycosides
34	315.1058	16.54	$C_{10}H_{14}N_6O_6$	$[M+H]^+$	100 %	2-Amino−8-(hydroxyamino)−9-pentofuranosyl−3,9-dihydro−6h-purin−6-one	72.34 %	Amino acids, peptides and analogues
35	595.1656	16.77	$C_{27}H_{30}O_{15}$	$[M+H]^+$	97.69 %	Nicotifolin	97.41 %	Flavonoid glycosides
40	449.1079	17.48	$C_{21}H_{20}O_{11}$	$[M+H]^+$	100 %	Cinaroside	96.98 %	Flavonoid glycosides
43	299.1108	20.39	$C_{12}H_{20}O_7$	$[M+Na]^+$	100 %	Triéthyl citrate	100 %	Fatty acid esters
48	241.0979	23.82	$C_{14}H_{12}N_2O_2$	$[M+H]^+$	100 %	9H-pyrido[3,4-*b*]indole−3-carboxylic acid ethyl ester	100 %	Pyridoindoles
51	202.087	25	$C_{12}H_{11}NO_2$	$[M+H]^+$	100 %	Ethyl quinoline−3-carboxylate	80.25 %	Fatty acid esters
54	343.2968	25.65	$C_{19}H_{38}N_2O_3$	$[M+H]^+$	100 %	Cocamidopropyl betaine	98.63 %	Amino acids, peptides and analogues
55	287.0913	25.84	$C_{16}H_{14}O_5$	$[M+H]^+$	100 %	Sakuranetin	97.66 %	O-methylated flavonoids
59	307.2246	25.8	$C_{17}H_{32}O_3$	$[M+Na]^+$	99.97 %	7-Oxoheptadecanoic acid/9-keto margaric acid	68.75 %	Fatty acids and conjugates
63	699.3566	26.16	$C_{33}H_{56}O_{14}$	$[M+Na]^+$	98.74 %	(2S)−3-[(6-O-β-L-glucopyranosyl-β-L-galactopyranosyl)oxy]−2-hydroxypropyl (9Z,12Z,15Z)-octadeca−9,12,15-trienoate	97.24 %	Glycosylglycerols
64	518.3235	26.23	$C_{26}H_{48}NO_7P$	$[M+H]^+$	100 %	1-alpha-Linolenoyl-glycero−3-phosphocholine (LysoPC(18:3(9Z,12Z,15Z)))	98.98 %	Glycerophosphocholines
65	476.2785	26.31	$C_{23}H_{42}NO_7P$	$[M+H]^+$	100 %	PE(18:3(6Z,9Z,12Z)/0:0)/PE(18:3(9Z,12Z,15Z)/0:0)/PE(0:0/18:3(6Z,9Z,12Z))/PE(0:0/18:3(9Z,12Z,15Z))	100 %	Amino acids, peptides and analogues
66	312.1132	26.53	$C_{20}H_{13}N_3O$	$[M+H]^+$	100 %	Pityriacitrin	80.00 %	Pyridoindoles
67	277.2162	26.53	$C_{18}H_{28}O_2$	$[M+H]^+$	100 %	10,15-Octadecadiynoic acid	71.43 %	Lineolic acids and derivatives
68	478.3376	26.53	$C_{24}H_{47}NO_8$	$[M+H]^+$	100 %	1-Glucopyranosyl−2-amino−3,17-dihydroxyoct−4-ène	87.10 %	Glycosphingolipids
69	301.1075	26.61	$C_{17}H_{16}O_5$	$[M+H]^+$	99.10 %	Naringenin 7,4′-Dimethyl Ether	100 %	O-methylated flavonoids
70	520.3417	26.74	$C_{26}H_{50}NO_7P$	$[M+H]^+$	100 %	2-linoleoyl-sn-glycero−3-phosphocholine (LysoPC (18:2(9Z,12Z)))	93.07 %	Glycerophosphocholines
71	496.3392	26.79	$C_{31}H_{58}O_{14}$	$[M+H]^+$	100 %	1–16:0-lysoPC	100 %	Glycerophosphocholines
72	522.3547	27.05	$C_{26}H_{52}NO_7P$	$[M+H]^+$	100 %	Lysolecithin	100 %	Glycerophosphocholines
73	291.233	27.3	$C_{19}H_{30}O_2$	$[M+H]^+$	100 %	cis-Parinaric acid methyl ester	88.75 %	Lineolic acids and derivatives
74	321.24	27.35	$C_{18}H_{34}O_3$	$[M+Na]^+$	95.22 %	12-Hydroxyoctadecanoic acid	88.81 %	Fatty acids and conjugates

extract, comprising 77 metabolites across 30 subclasses. This compositional profile is consistent with prior analyses of *S. tuberosa* leaves, which likewise reported phenolic/flavonoid predominance and specifically identified rutin, quercetin, and ellagic acid as major constituents; those studies also linked these metabolites to antioxidant and antimicrobial effects (da Silva et al., 2012; Thassya Lucas Dos Santos et al., 2019). Moreover, the limited intrinsic antifungal activity but potentiation of fluconazole and inhibition of *Candida* morphogenesis seen with *S. tuberosa* leaf extracts (Thassya Lucas Dos Santos et al., 2019) align with our non-bactericidal, anti-virulence profile against *P. aeruginosa*, reinforcing the relevance of phenolic-rich fractions as modulators of pathogen fitness rather than direct killers. Among these compounds, flavonoids were prominent, including rutin, a compound previously reported to interfere with QS and biofilm formation in *Escherichia coli* (Peng et al., 2018). However, rutin at 64 $\mu g \cdot mL^{-1}$ did not significantly affect pyocyanin production by *P. aeruginosa* in our model (data not shown), suggesting that the anti-virulence activity of the STF extract is likely attributable to other constituents or to synergistic interactions among multiple compounds, rather than to rutin alone. In contrast, related glycosides such as nicotifolin and cinaroside have not yet been studied in this context, making them attractive candidates for further exploration. The presence of O-methylated flavonoids such as sakuranetin and naringenin dimethyl ether, whose role in bacterial communication remains unknown, highlights the presence of structurally diverse compounds with unexplored bioactivity deserving further investigation. Other compound classes likely contributed to the observed anti-QS effect. Carbohydrates and their conjugates (14.3 %), including cerelose and complex glycosides, may mimic or disrupt sugar-based autoinducer signaling pathways. Carboxylic acid derivatives (13 %) such as hibiscus acid methyl ester and hydroxycitric acid dimethyl ester, known to stimulate metabolism in fungi (Sumang et al., 2024), could also interfere with bacterial communication or metabolism. Amino acid derivatives like methyl and ethyl pyroglutamate may disrupt protein synthesis or regulatory proteins involved in QS. Hydroxycinnamic acids, particularly ethyl caffeate, are recognized for antioxidant activity, and similar compounds have also been linked to QS inhibition (Shariati et al., 2024). Fatty acids such as linoleic acid and 10,15-octadecadiynoic acid may alter bacterial membrane integrity or

Table 3
UPLC-HRMS data obtained after analysis of the STF extract, listing compounds identified in negative ionization mode. The table includes retention time (RT), mass-to-charge ratio (*m/z*), molecular formula, adduct type, identification score, proposed molecular structure, similarity index, and chemical subclass. The identified metabolites belong to diverse biochemical classes, including carbohydrates, amino acids, flavonoids, fatty acids, and phospholipids, offering insights into the chemical composition and potential bioactivity of the extract.

N°	*m/z*	RT (min)	Mass formula	Adduct	Score	Proposed structure	Similarity	Subclass
1	317.0551	0.4	$C_9H_{18}O_{10}S$	$[M-H]^-$	99.94 %	6-Sulfo-quinovosyl-Glycerin	73.71 %	Carbohydrates and carbohydrate conjugates
4	203.0191	0.52	$C_7H_8O_7$	$[M-H]^-$	100 %	Hibiscus acid 1-methylester	76.73 %	Tricarboxlylic acids and derivatives
5	189.0037	0.65	$C_6H_6O_7$	$[M-H]^-$	99.99 %	Hibiscus lactone	99.44 %	Tricarboxlylic acids and derivatives
6	235.0451	0.71	$C_8H_{12}O_8$	$[M-H]^-$	99.99 %	2-(2-Ethoxy−2-oxoethyl)−2-hydroperoxybutanedioic acid	70.68 %	Tricarboxlylic acids and derivatives
8	315.0727	0.86	$C_{13}H_{16}O_9$	$[M-H]^-$	89.13 %	2,5-dihydroxybenzoic acid 5-*O*-β-D-glucoside	98.91 %	Carbohydrates and carbohydrate conjugates
9	235.0457	0.86	$C_8H_{12}O_8$	$[M-H]^-$	98.36 %	(2 R,3S)−2-Hydroxycitric acid 3,5-dimethyl ester	71.81 %	Dicarboxylic acids and derivatives
11	323.1335	1.52	$C_{13}H_{24}O_9$	$[M-H]^-$	100 %	2,2-Dimethyl−4beta-[(beta-D-glucopyranosyloxy) methyl]−1,3-dioxolane−5-alpha (ou beta)-methanol	79.43 %	Fatty acyl glycosides
12	249.0605	1.77	$C_9H_{14}O_8$	$[M-H]^-$	100 %	2-Hydroxy−2-[2-(3-hydroxypropoxy)−2-oxoethyl] butanedioic acid	64.86 %	Carboxylic acids derivatives
13	249.061	2.07	$C_9H_{14}O_8$	$[M-H]^-$	100 %	erythro-Pentaric acid, 2-deoxy−3-C-(methoxycarbonyl)-, dimethyl ester	77.65 %	Tricarboxlylic acids and derivatives
14	249.061	2.14	$C_9H_{14}O_8$	$[M-H]^-$	100 %	2-Hydroxy−2-[2-(2-methoxyethoxy)−2-oxoethyl] butanedioic acid	72.97 %	Tricarboxlylic acids and derivatives
16	327.1081	4.63	$C_{15}H_{20}O_8$	$[M-H]^-$	98.84 %	3-(4-hydroxyphenyl)−3-oxopropyl β-D-glucopyranoside	97.29 %	Carbonyl compounds
18	263.0768	5.7	$C_{10}H_{16}O_8$	$[M-H]^-$	99.92 %	2-Hydroxy−2-[2-(4-hydroxybutoxy)−2-oxoethyl] butanedioic acid	64.14 %	Carboxylic acids derivatives
19	263.0771	5.8	$C_{10}H_{16}O_8$	$[M-H]^-$	100 %	2-Hydroxy−2-[2-(4-hydroxybutoxy)−2-oxoethyl] butanedioic acid	68.91 %	Carboxylic acids derivatives
20	263.0764	5.86	$C_{10}H_{16}O_8$	$[M-H]^-$	100 %	2-Hydroxy−2-[2-(4-hydroxybutoxy)−2-oxoethyl] butanedioic acid	70.97 %	Carboxylic acids derivatives
21	133.0285	6.1	$C_8H_6O_2$	$[M-H]^-$	100 %	4-éthynolbenzenol	72.57 %	1-hydroxy−2-unsubstituted benzenoids
22	359.0981	6.61	$C_{15}H_{20}O_{10}$	$[M-H]^-$	94.31 %	[3,4,5-Trihydroxy−6-(hydroxymethyl)oxan−2-yl] 3-hydroxy−4,5-dimethoxybenzoate	99.01 %	Hydrolysable tannins
23	325.0925	6.69	$C_{15}H_{18}O_8$	$[M-H]^-$	99.93 %	1-p-Coumarylglucose	94.83 %	Hydroxycinnamic acid esters
24	355.1029	8.08	$C_{16}H_{20}O_9$	$[M-H]^-$	99.99 %	1-*O*-feruloyl-β-D-glucose	96.67 %	Hydroxycinnamic acids and derivatives
25	447.1511	8.54	$C_{19}H_{28}O_{12}$	$[M-H]^-$	77.51 %	(2 R,3S,4 R,5S)−2-(hydroxymethyl)−6-[phenyl-[(3S,4 R,5S,6 R)−3,4,5-trihydroxy−6-(hydroxymethyl)oxan−2-yl]oxymethoxy] oxane−3,4,5-triol	76.02 %	Carbohydrates and carbohydrate conjugates
26	447.15	8.95	$C_{19}H_{28}O_{12}$	$[M-H]^-$	98.40 %	2-[hydroxy(phenyl)methoxy]−6-({[3,4,5-trihydroxy−6-(hydroxymethyl)oxan−2-yl]oxy} methyl)oxane−3,4,5-triol	83.40 %	Carbohydrates and carbohydrate conjugates
28	197.045	9.64	$C_9H_{10}O_5$	$[M-H]^-$	100 %	Phyllemblin	100 %	Benzoic acids and derivatives
30	439.182	13.66	$C_{18}H_{32}O_{12}$	$[M-H]^-$	90.45 %	6-O-Hexanoyl-alpha,alpha-D-trehalose	61.94 %	Fatty acyls
31	433.2068	13.74	$C_{20}H_{34}O_{10}$	$[M-H]^-$	99.91 %	Famille des alkylglycosides	76.51 %	Fatty acyls
32	609.1445	14.98	$C_{27}H_{30}O_{16}$	$[M-H]^-$	92.76 %	Rutine	100 %	Flavonoid glycosides
33	413.1808	16.03	$C_{20}H_{30}O_9$	$[M-H]^-$	99.86 %	Ptesculentoside	73.95 %	Sesquiterpenoids
35	593.151	16.74	$C_{27}H_{30}O_{15}$	$[M-H]^-$	83.98 %	Nicotifolin	95.79 %	Flavonoid glycosides
36	593.151	16.81	$C_{27}H_{30}O_{15}$	$[M-H]^-$	89.22 %	Kaempferol 3-neohesperidoside	90.44 %	Flavonoid glycosides
37	447.0922	17.04	$C_{22}H_{16}N_4O_7$	$[M-H]^-$	77.53 %	Trifolin	100 %	Flavonoid glycosides
38	503.1769	17.11	$C_{22}H_{32}O_{13}$	$[M-H]^-$	100 %	Coniferinoside	76.71 %	Carbohydrates and carbohydrate conjugates
39	417.2121	17.24	$C_{20}H_{34}O_9$	$[M-H]^-$	100 %	(5 R,8Z,11Z)−5-(beta-D-Glucopyranosyloxy)−6-hydroxy−8,11-tetradecadienoic acid	60.93 %	Carbohydrates and carbohydrate conjugates
41	615.0981	17.67	$C_{28}H_{24}O_{16}$	$[M-H]^-$	100 %	Quercétin 3-(2‴galloylglucoside)	100 %	Flavonoid glycosides
42	503.1763	17.73	$C_{22}H_{32}O_{13}$	$[M-H]^-$	99.89 %	Coniferinoside	76.25 %	Carbohydrates and carbohydrate conjugates
44	207.0656	21.07	$C_{11}H_{12}O_4$	$[M-H]^-$	100 %	Ethyl caffeate	97.95 %	Hydroxycinnamic acids and derivatives
45	355.1182	22.08	$C_{20}H_{20}O_6$	$[M-H]^-$	99.28 %	Dimethyl 2,4-bis(4-hydroxyphenyl)cyclobutane−1,3-dicarboxylate	80.54 %	Lignans, neolignans ans related compounds
46	455.1914	22.94	$C_{22}H_{32}O_{10}$	$[M-H]^-$	98.61 %	Glycoconjugué	66.25 %	Carbohydrates and carbohydrate conjugates
47	191.0708	23.62	$C_{11}H_{12}O_3$	$[M-H]^-$	99.55 %	p-coumaric acid ethyl ester	100 %	Hydroxycinnamic acids and derivatives
49	242.1755	24.28	$C_{13}H_{25}NO_3$	$[M-H]^-$	90.18 %	13-Amino−13-oxotridecanoic acid	68.94 %	Amino acids, peptides and analogues

(*continued on next page*)

Table 3 (*continued*)

N°	*m/z*	RT (min)	Mass formula	Adduct	Score	Proposed structure	Similarity	Subclass
50	188.0709	24.68	$C_{11}H_{11}NO_2$	[M-H]⁻	100 %	Lindole−3-éthyl formiate	97.03 %	Indolecarboxylic acids and derivatives
52	399.1446	25.21	$C_{22}H_{24}O_7$	[M-H]⁻	100 %	(10-acetyloxy−3,8,8-trimethyl−2-oxo−9,10-dihydropyrano[2,3-*f*]chromen−9-yl) (*Z*)−2-methylbut−2-enoate	63.35 %	1-hydroxy−2-unsubstituted benzenoids
53	423.1302	25.53	$C_{20}H_{24}O_{10}$	[M-H]⁻	100 %	{3,4,5-trihydroxy−6-[(3-phenylprop−2-enoyl)oxy] oxan−2-yl}methyl 3,4-dihydroxy−2-methylidenebutanoate	70.92 %	Hydroxycinnamic acids and derivatives
55	285.0766	25.67	$C_{16}H_{14}O_5$	[M-H]⁻	100 %	Sakuranetin	97.27 %	O-methylated flavonoids
56	474.2622	25.76	$C_{23}H_{42}NO_7P$	[M-H]⁻	84.13 %	1–18:3-lysoPE	92.22 %	Amino acids, peptides and analogues
58	283.1372	25.76	$C_{15}H_{24}O_3S$	[M-H]⁻	100 %	Nonylbenzenesulfonic acid	87.82 %	Benzenesulfonic acids and derivatives
57	593.2746	25.76	$C_{27}H_{47}O_{12}P$	[M-H]⁻	84.80 %	1-acylglycerophosphoinositol/PI(18:3(9Z,12Z,15Z)/ 0:0)	95.19 %	Glycerophosphoinositols
61	297.1536	25.99	$C_{16}H_{26}O_3S$	[M-H]⁻	99.72 %	4-decylbenzenesulfonate	93.46 %	Benzenesulfonic acids and derivatives
60	571.2892	25.99	$C_{25}H_{49}O_{12}P$	[M-H]⁻	100 %	1-palmitoylglycerophosphoinositol	100 %	Glycerophosphoinositols
62	265.1488	25.99	$C_{12}H_{26}O_4S$	[M-H]⁻	99.96 %	dodecyl sulfate	100 %	Sulfuric acid esters

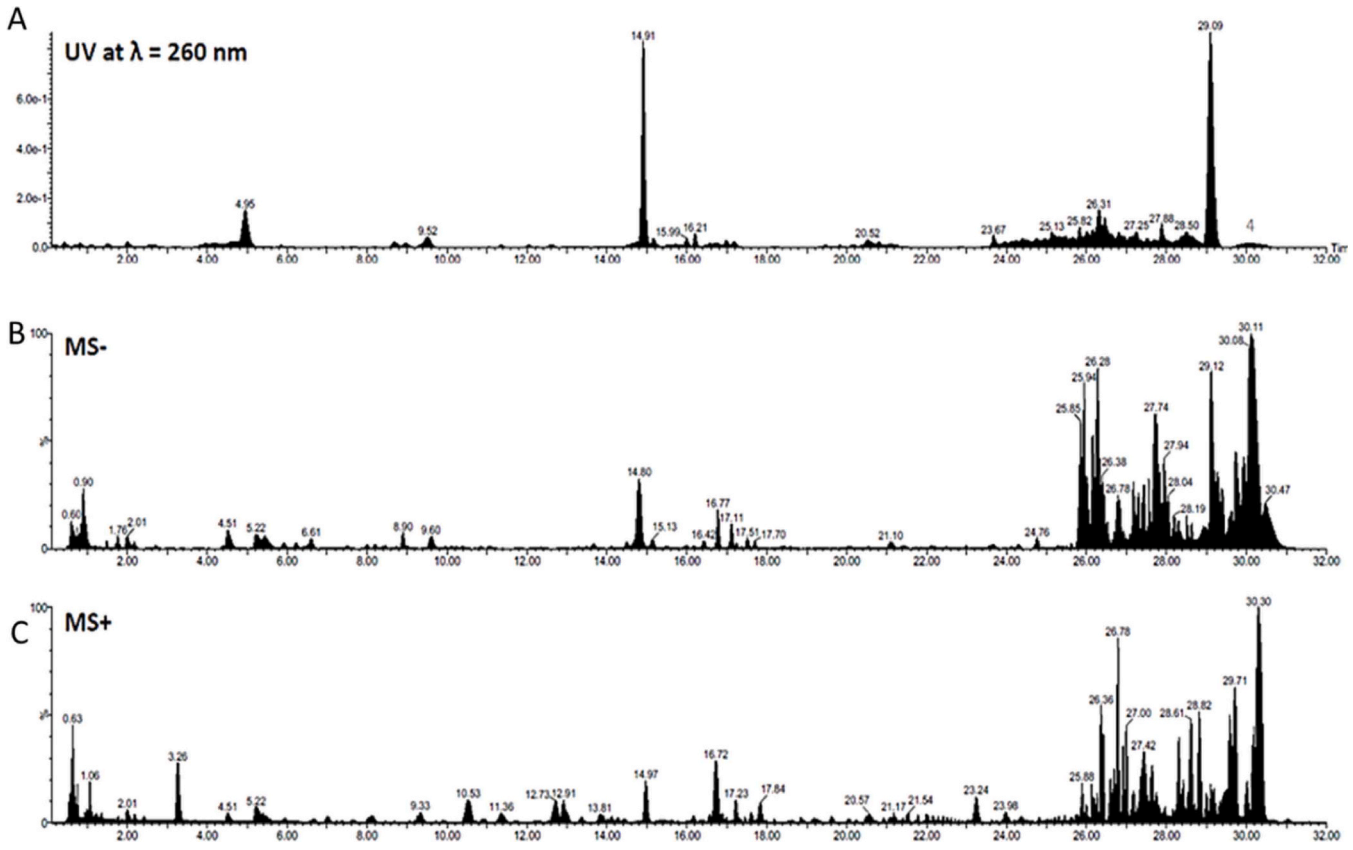


Fig. 4. UV and MS chromatograms of the STF extract. (A) UV chromatogram recorded at 260 nm; (B) MS chromatogram in negative ionization mode (MS^-); (C) MS chromatogram in positive ionization mode (MS^+). Chromatograms show the distribution and intensity of compounds detected, illustrating the complexity of the STF extract composition.

interfere with DSF-type signaling systems (Kim et al., 2021), suggesting possible cross-talk mechanisms. Additional compounds, including the benzenediol loliolide, lysolecithins, and the β-carboline alkaloid harman-3-carboxylic acid, offer further mechanistic hypotheses. Loliolide has demonstrated broad antimicrobial activity (Morais et al., 2024), while lysolecithins, being amphipathic, could indirectly affect QS through membrane destabilization. Harman-3-carboxylic acid, although not directly linked to QS, may possess unexplored bioactivity relevant to bacterial signaling. Together, these structurally varied molecules likely exert their effects through complementary and possibly synergistic mechanisms. While flavonoids and hydroxycinnamic acids appear central to QS modulation, other families—including carboxylic acids, fatty acids, and amino acid derivatives—may enhance the overall antimicrobial potential of the extract. Importantly, most of these compounds have not been characterized for their impact on bacterial QS, underscoring the originality and potential of STF as a reservoir of novel QS inhibitors. These findings are particularly relevant in the context of early-stage infections, where QS-regulated virulence factors are essential for bacterial colonization and immune evasion. Moreover, although the potential synergy between STF and conventional antibiotics was not

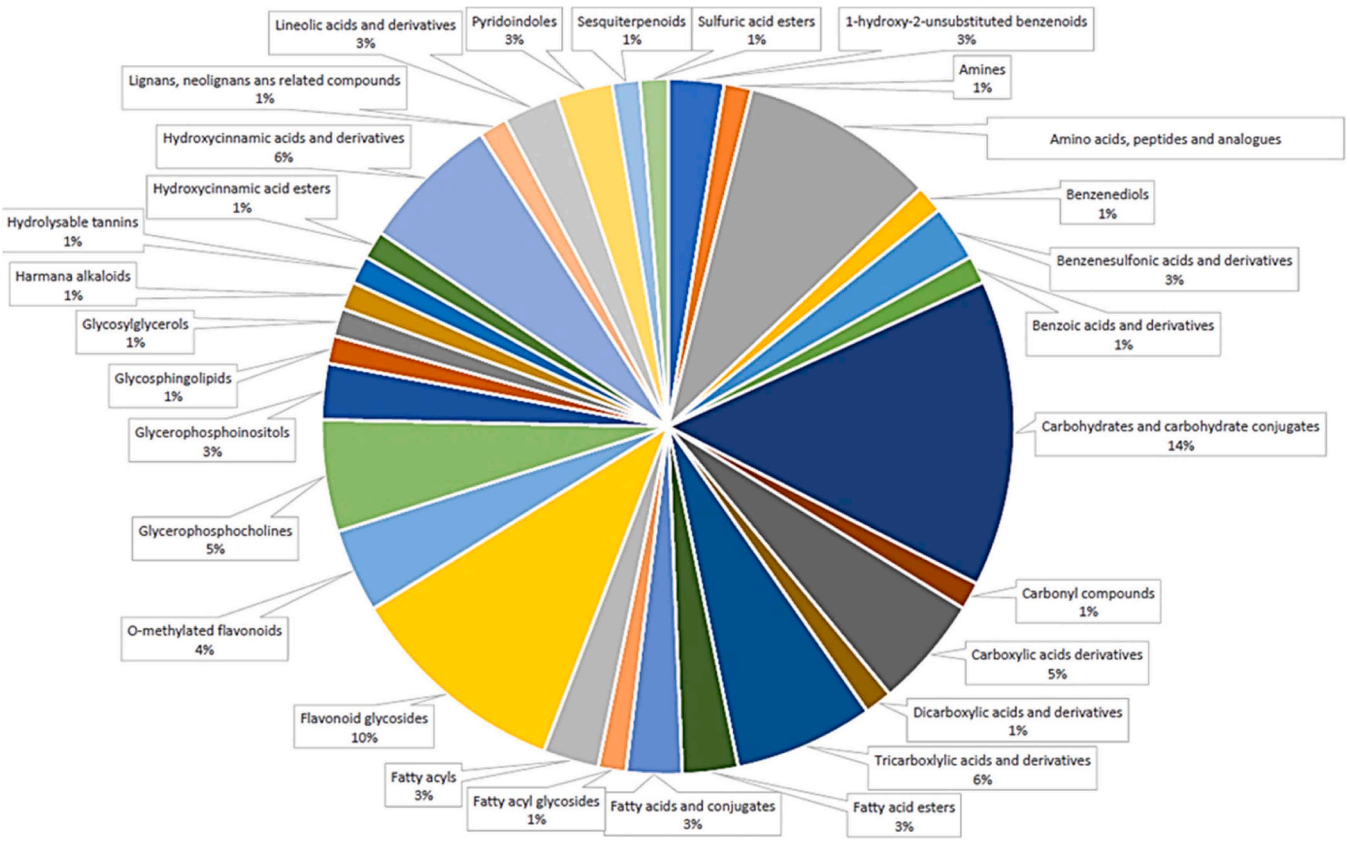


Fig. 5. Chemical subclass distribution of metabolites identified in the STF leaf extract by UHPLC-HRMS. Pie chart representing the relative proportions of the 77 identified compounds in STF leaf extract, highlighting predominant subclasses: carbohydrates and carbohydrate conjugates (14 %), flavonoid glycosides (10 %), carboxylic acid derivatives (5 %), glycerophosphocholines (5 %), and hydroxycinnamic acid derivatives (6 %).

explored in this study, the extract's non-bactericidal profile makes it a promising candidate for combination therapies, which have been shown to restore antibiotic susceptibility in resistant *P. aeruginosa* strains (Wang et al., 2024). Finally, STF is derived from *Spondias tuberosa*, a plant endemic to the Caatinga biome. This highlights the pharmacological richness of underexplored ecosystems and the value of bioprospecting efforts grounded in traditional knowledge. Such initiatives not only support innovative anti-infective strategies but also contribute to the sustainable valorization of local biodiversity in relation to their use for traditional medicine as previously reported (de Oliveira-Júnior et al., 2018).

5. Conclusion

This study demonstrates that *Spondias tuberosa* Arruda ethanolic leaf extract effectively inhibits pyocyanin production and disrupts QS in planktonic *P. aeruginosa* without affecting bacterial growth. These findings underscore the potential of STF as a source of anti-virulence compounds and open perspectives for further research into its bioactive molecules, synergistic applications with antibiotics, and possible therapeutic development.

CRediT authorship contribution statement

S. Chevalier: Investigation, Funding acquisitions. **K. Baranger**: Investigation, Funding acquisitions. **L. Quintans-Junior**: Investigation. **J.R. Guedes da Silva Almeida**: Investigation. **R. Gonçalves de Oliveira Junior**: Investigation. **P. Sitarek**: Investigation **R. Grougnet**: Investigation. **A. Tahrioui**: Investigation. **O. Lesouhaitier**: Investigation. **M. Barreau**: Investigation. **P.E. Bodet**: Investigation. **A.S. Tareau**: Investigation. **Picot L.**: Writing – original draft, Investigation, Data curation, Conceptualization.

Declaration of Competing Interest

The authors declare they have no conflict of interests.

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Data availability

Data will be made available on request.

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