



Light-driven bacterial inactivation using *Plumeria rubra* extract: a promising natural photosensitizer

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Abstract

Antimicrobial Photodynamic Inactivation (aPDI) is a non-thermal, minimally invasive technique that uses light and photosensitizing compounds (PS) to generate reactive oxygen species, promoting the destruction of microorganisms without inducing microbial resistance. This study investigated the photosensitizing potential of the flower extract of *Plumeria rubra* L., an unconventional edible plant, in aPDI. The extract showed a high content of rutin, a flavonoid known to confer chemical stability to PS and promote its interaction with bacterial cell structures, increasing photodynamic efficiency. Tests conducted in the dark revealed a lack of antimicrobial activity, a desirable characteristic for photosensitizing compounds. Under white light irradiation and an optical fluence of 18 J/cm², the extract inactivated the methicillin-resistant *Staphylococcus aureus* strain at a concentration of 0.78 mg/mL, in addition to demonstrating action against *Listeria monocytogenes* and *Staphylococcus epidermidis*. At an optical fluence of 27 J/cm², inactivation of most of the strains evaluated was observed. These results indicate that *P. rubra* extract it is a promising natural photosensitizer, that combines a favorable phytochemical composition, absence of inactivation in the dark, and high photodynamic efficiency under white light, demonstrating potential for therapeutic and industrial applications.

Keywords Non-conventional edible plants · Antimicrobial photodynamic therapy · Plant-derived compounds · Bioactive extracts

Introduction

Photodynamic inactivation (PDI) can be understood as a non-thermal and minimally invasive technique that uses light and photosensitizing compounds (PS) to generate reactive oxygen species (ROS) that interact with cells and microorganisms, leading to their death. It emerges as an auxiliary or alternative technique to traditional preservation or treatment methods, offering broad-spectrum antimicrobial activity that does not generate microbial resistance (Henderson and Dougherty 1992; Dougherty et al. 1998; Castano et al. 2004; Hamblin 2016; Pang et al. 2025).

PDI is based on three main pillars: a non-toxic compound that is activated by light (PS); a light source with an emission wavelength that matches the optical absorption band of PS; and the presence of oxygen in the target tissue or cellular environment (Dougherty et al. 1998; Castano et al. 2004).

The PDI mechanism of action begins with the absorption of light by PS in the ground singlet state (⁰PS), which

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transitions to the excited and unstable singlet state (^1PS). Through intersystem crossing, ^1PS converts to the triplet state (^3PS), which has a longer lifetime. From this point on, the PDI mechanism can follow two main reactions that generate ROS. In the Type I reaction, ^3PS reacts with substances present in the surrounding medium via electron or hydrogen transfer, resulting in the formation of free radicals, including superoxide anion, hydrogen peroxide, and hydroxyl radicals, which cause oxidative damage to cells. In the Type II reaction, ^3PS transfers energy directly to oxygen in the triplet state ($^3\text{O}_2$), converting it to the highly reactive singlet state ($^1\text{O}_2$), which, like other ROS, generates cellular damage and induces cell death. Figure 1 presents the aPDI mechanism through a simplified diagram (Henderson and Dougherty 1992; Dougherty et al. 1998; Castano et al. 2004; Hamblin 2016).

PDI, including the antimicrobial modality (aPDI), is a minimally invasive approach with broad and expanding applications. They are considered a relevant strategy against bacterial resistance, also demonstrating efficacy in inactivating some fungi, viruses, parasites, and microbial biofilms. When used to combat disease, it is more commonly referred to as Photodynamic Therapy (PDT). It has been used in oncology for the treatment of solid tumors and precancerous lesions, in ophthalmology for the treatment of retinal macular degeneration, in dermatology for reducing acne and fungal infections, and in dentistry for oral disinfection and the treatment of periodontitis (Hamblin 2016; Nayak et al. 2025; Fiegler-Rudol et al. 2025; Pang et al. 2025; Qureshi et al. 2024).

PDI/aPDI/PDT is a promising therapeutic approach, in which the efficacy depends heavily on the appropriate selection of PS. Conventional synthetic PS, such as first- and second-generation porphyrins, have clinical limitations due to toxicity, limited availability, and difficulty in controlling cellular damage. In this context, it is essential to explore PS of natural origin, recognized for their safety, low toxicity, abundance, and high biocompatibility (Aziz et al. 2023; Wang et al. 2023).

The structural diversity of natural PS confers selectivity and specific biological activities, making them promising alternatives that may be more effective than synthetic

analogues. Therefore, the investigation of natural PS constitutes a strategic avenue for improving aPDI/PDT, offering safer and more sustainable therapies that can address clinical challenges such as antimicrobial resistance (Aziz et al. 2023; Wang et al. 2023; Aebisher et al. 2024; Zhou et al. 2024).

Unconventional Edible Plants (UEP) are a viable alternative source to contribute to the search for new natural PS. UEP are commercially underexploited edible plant species that are not part of the usual diet but possess nutritional, functional, or bioactive value. The exploitation of PS in UEP offers several advantages: they can provide natural pigments such as anthocyanins, carotenoids, chlorophylls, and betalains, which, in addition to imparting color to foods, cosmetics, or therapeutic products, often exhibit biological activities such as antioxidant, antimicrobial, or photosensitizing action. Bioprospecting these plants also adds economic value to underutilized species, encourages the cultivation and preservation of local biodiversity, and enables the development of innovative functional products that combine nutritional, therapeutic, and environmental benefits in a single natural resource (Leal et al. 2018; Milião et al. 2022; Ferreira et al. 2024).

In this work, we report the methodology and results of evaluating the extract from the flowers of UEP *Plumeria rubra* L. (*P. rubra*) as a candidate for PS in aPDI for the elimination of bacteria causing foodborne diseases and of clinical interest.

P. rubra (Family: Apocynaceae) is native to Central America, northern South America, and Mexico, and is now widely distributed in regions such as the Caribbean, South America, Africa, China, and the Pacific. It is an easy-to-grow plant that can thrive in a variety of soil types, including clay and sandy soils, as well as acidic and alkaline conditions. However, it performs best in well-drained soils. Gilman and Watson (1994); Vahl (2012); Acevedo-Rodríguez and Strong (2012).

P. rubra is widely cultivated as an ornamental plant in gardens and public spaces, characterized by salpiform flowers with variable colors, including white, yellow, pink, red, or spots with shades Fig. 2 shows a photograph of *P. rubra* flowers. Gilman and Watson (1994); Rojas-Sandoval (2024)

Fig. 1 Simplified representation of the mechanism of action of PDI: Light absorption by ^0PS and transition from the ^1PS state, decay by intersystem crossing to the ^3PS state, followed by two paths: transfer of electrons or hydrogen to biomolecules generating free radicals (Type I reaction) or energy transfer from ^3PS to $^3\text{O}_2$ generating $^1\text{O}_2$ (Type II reaction), in both the ROS generated causes cell death

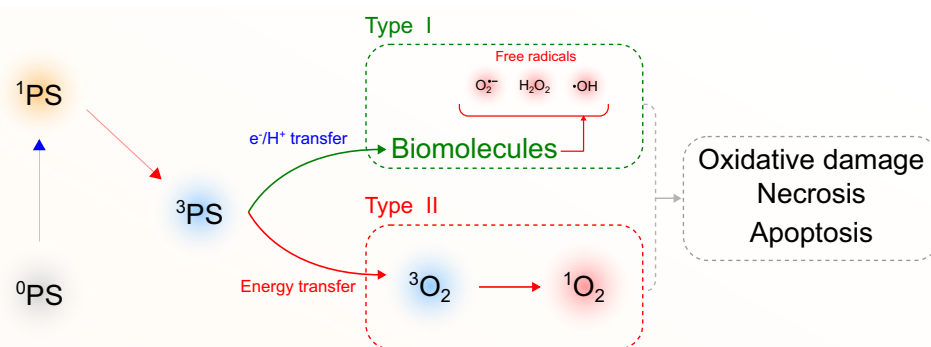




Fig. 2 Photograph of the flowers of the *P. rubra*. Image reproduced from Rojas-Sandoval, CC BY-SA 3.0 license (Rojas-Sandoval 2024)

P. rubra exhibits phytochemical diversity marked by anthocyanins, iridoids, triterpenes, flavonoids, as well as phenolic acids, coumarins, aromatic esters, and phytosterols. This composition reflects the bioactive potential of this specie, associated with specific anti-inflammatory, antioxidant, and pharmacological properties. Byamukama et al. (n.d.); Sharma et al. (2011); Isa et al. (2018); Bihani (2021); Malaspina et al. (2024); Calva and Celi (2024)

Widely used in traditional medicine in pantropical regions, *P. rubra* has diverse therapeutic applications. In South America, decoctions of leaves, bark, and heartwood are used as anthelmintics, purgatives, and emmenagogues, as well as in topical preparations for skin diseases. In East Asia, the fruit pulp, latex, and stem bark are traditionally used as abortifacients and purgatives. In addition to its medicinal value, the flowers of this species are used in the preparation of sweets, jellies, and salads (Hamburger et al. 1991; Sharma et al. 2011; Kalantri and Aher 2016).

To evaluate the potential of *P. rubra* flowers to provide a natural PS, the flower extract was obtained via ultrasound-assisted alcoholic extraction, followed by the characterization of the phenolic compound profile, antioxidant activity, antibacterial activity, light absorption in the visible region of the electromagnetic spectrum and capacity for photodynamic bacterial inactivation in vitro, determining the light dosimetry and concentration required for this purpose.

Materials and methods

Obtaining the extract

Fresh flowers of *P. rubra* were collected by hand, cleaned with distilled water to remove impurities, and dried in an oven with air circulation at 40–45 °C for approximately 48

hours, until they reached a constant weight. The dried flowers were then ground in a knife mill to a fine powder. The material was stored in vacuum-sealed plastic bags at a temperature of -18 °C until the extract was obtained.

The aqueous extract of *P. rubra* flowers was obtained by hydroalcoholic extraction (70% v/v) assisted by ultrasound (Elma, Elmasonic P) at a frequency of 80 kHz and a power of 100 W for 60 minutes. Subsequently, the extracts were concentrated in a rotary evaporator (Fisatom, Model 804) until reaching approximately 20% of the initial volume and then lyophilized (Labconco, Freeze Zone 6) at a temperature of -50 °C and an absolute pressure of less than 0.8 mBar, undergoing a heat exchange process with a heating source at 40 °C for 40 h or until completely dry. The dyes were packaged in hermetically sealed jars and stored at 4 °C for future analysis.

The choice of the ultrasound-assisted hydroalcoholic solution extraction method was based on the advantages associated with this technique. Ethanol, used as a solvent, is considered safe, non-toxic, easily obtained, comes from a renewable source, and has a high affinity for phenolic compounds. Furthermore, ultrasound-assisted extraction, due to the phenomenon of acoustic cavitation, generates shock waves capable of rupturing the cell walls of the plant material. This process increases the contact area between the matrix and the solvent, thereby favoring the diffusion of the target compounds and enabling extraction in less time, at moderate temperatures, with reduced energy and solvent consumption (Kumar et al. 2021; Cui et al. 2022; Silva et al. 2023; Athirojthanakij and Rashidinejad 2024; Sethi and Rathod 2025).

Phenolic profile

Phenolic compounds were analyzed using a UHPLC system (Thermo Scientific, Dionex Ultimate 3000) equipped with a diode array detector coupled in series to a high-resolution mass spectrometer (Orbitrap Exploris 120, Thermo Finnigan). Separation was performed on a Luna C18 column (3 $\mu\text{m} \times 4.6 \times 150$ mm) maintained at 35 °C (Phenomenex, Torrance). Chromatographic conditions used for compound separation were established as described in detail by Gonçalves et al. (2024).

For the mass spectrometer, heated electrospray ionization (H-ESI) in negative mode (2500 V) was employed. Nebulizing gas (N_2) was supplied at a flow rate of 50 a.u., with auxiliary gas at 10 a.u. and sweep gas at one a.u. The ion transfer tube and vaporizer temperatures were set at 325 °C and 350 °C, respectively. Data-dependent acquisition mode was used with a normalized collision energy of 30%. Mass spectra were recorded in the m/z range of 110–1100, with a resolution of 60,000 for MS and 17,000 for MS/MS.

Compound identification was based on UV–Vis spectra, accurate mass, molecular formula, and fragmentation pattern, compared with authentic standards or literature data. Quantification of phenolic compounds was performed using external calibration curves of the respective standards or, when unavailable, of the most structurally similar compound.

Antioxidant activity

A PS with high antioxidant activity in the absence of light can exhibit greater chemical stability and also contribute to reducing its basal toxicity (in the dark), thereby minimizing potential side effects on tissues before light application. Based on this, the extract from *P. rubra* flowers was subjected to the Thiobarbituric Acid Reactive Substances (TBARS) assay to evaluate its antioxidant activity.

The TBARS assay was adapted to evaluate the antioxidant potential of *P. rubra* flower extract. The method is based on the reaction between lipid peroxidation products, such as malondialdehyde (MDA), and thiobarbituric acid (TBA), forming a complex that can be quantified spectrophotometrically (Ghani et al. 2017; Christodoulou et al. 2022).

The experimental procedure used porcine brain homogenate in Tris-HCl buffer, to which ascorbic acid (0.1 mM, 0.1 mL), ferrous sulfate (10 μ M, 0.1 mL), and different concentrations of *P. rubra* extract were added. After incubation at 37 °C to induce peroxidation, trichloroacetic (28 % w/v, 0.5 mL) acid and TBA (2% w/v, 0.38 mL) were added, followed by further incubation at 80 °C. The samples were then centrifuged, and the supernatant was analyzed for absorbance at 532 nm.

The analysis of antioxidant activity, as measured by the TBARS assay, was performed by relating the absorbance at 532 nm to the extract concentration, using a logistic model to adjust the data, as described in Eq. 1.

$$A_{532}(C) = A_2 + \frac{(A_1 - A_2)}{\left[1 + \left(\frac{C}{EC_{50}}\right)^p\right]} \quad (1)$$

Applied to the TBARS technique, the parameters of the logistic model, given by Eq. 1, can be described as: A_1 being the absorbance value without extract, such that oxidation is maximum, that is, without antioxidant action; A_2 being the absorbance at a high concentration of the extract, corresponding to minimum oxidation; EC_{50} being the concentration that reduces oxidation by 50%, that is, the lower the EC_{50} , the greater the antioxidant action of the extract; and the parameter p , known as the Hill coefficient, here denoted as the slope parameter and which indicates how oxidation inhibition evolves with extract concentration.

Antibacterial activity

It is desirable that the PS does not exhibit antimicrobial activity in the absence of light, or that the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) values are high under these conditions. Before performing the aPDI assay, the antimicrobial activity of the candidate PS was evaluated in the absence of light. For this purpose, MIC and MBC were determined and also served as references to establish the concentrations of the *P. rubra* flower extract used in the aPDI assay.

The efficacy of *P. rubra* extract, both in the antimicrobial activity assay and in the aPDI assay, was evaluated against the following bacteria: *Cutibacterium acnes* ATCC 11827 (*C. acnes*), clinically isolated *Enterococcus faecalis* (*E. faecalis* CI), *Listeria monocytogenes* ATCC 19111 (*L. monocytogenes*) and clinically isolated *Listeria monocytogenes* (*L. monocytogenes* CI), clinically isolated *Morganella morganii* (*M. morganii* CI), clinically isolated methicillin-resistant *Staphylococcus aureus* (MRSA CI), *Pseudomonas aeruginosa* ATCC 9027 (*P. aeruginosa*), *Staphylococcus aureus* ATCC 25923 (*S. aureus*) and *Staphylococcus epidermidis* ATCC 12228 (*S. epidermidis*).

The bacterial strains were cultured in Petri dishes and incubated for 24 h at 37 °C to obtain viable bacteria at the maximum growth rate (*C. acnes* and *S. epidermidis* were incubated under anaerobic conditions). Then, using a McFarland densitometer, 10 mL of inocula were obtained in Muller-Hinton medium containing 10⁶ CFU/mL. These inocula were placed in a 96-well plate containing different concentrations (obtained by serial dilution) of *P. rubra* flower extract (from 100 mg/mL to 0.1 mg/mL), totaling 90 μ L per well. Subsequently, 10 μ L of the inoculum was added to each well to obtain a concentration of 10⁵ CFU/mL. The plates were incubated at 37 °C for 24 hours.

After the incubation period, 100 μ L of an aqueous solution containing 0.5 mg/mL of iodinitrotetrazolium (INT), which acted as a revealing agent for the bacterial metabolic activity, was added to each well. After four hours, it was verified that the wells containing the lowest concentration of the *P. rubra* extract did not show a color change, indicating the MIC. An aliquot of 10 μ L from the remaining wells, which also showed no change in color, was transferred to Petri dishes containing solid Muller-Hinton medium and incubated at 37 °C for 24 h to determine the MBC.

UV-VIS spectroscopy

To determine the ideal light source for the aPDI assay, the absorbance spectra of the *P. rubra* flower extract diluted in water were determined as a function of concentration. This analysis was performed using a spectrophotometer

capable of reading in 96-well plates (SpectroStar Nano, BMG Labtec). Starting from a concentration of 30 mg/mL and using the serial dilution procedure in a 96-well plate, spectra were acquired in the 400–700 nm region, with a 2 nm interval, for the aqueous extract at concentrations ranging from 30.00 mg/mL to 0.23 mg/mL (eight concentrations).

Light source

To ensure uniform light intensity across the surface of a 96-well plate, an LED-based lighting system was developed. The system consisted of 20 LEDs distributed evenly across an aluminum printed circuit board (PCB), coupled with a forced ventilation system to optimize heat dissipation.

The PCB-LED array was positioned on the underside of a rectangular tube whose inner walls were lined with mirrored acrylic sheets to increase efficiency and direct light emission to the top surface of the plate. A sandblasted acrylic sheet measuring 130 × 90 mm was placed on top to promote uniform light distribution. All components were integrated into a black acrylic housing.

The system's irradiation intensity (W/cm^2) was regulated by a microcontroller and calibrated using a spectroradiometer (Gooch & Housego; OL 756) coupled to an optical fiber (Gooch & Housego, OL 730 7q-1.0) and a 50.8 mm diameter integrating sphere (Gooch & Housego, IS-270).

Antimicrobial photodynamic inactivation assay

The aPDI assay was performed, as previously described (Herculano et al. 2026), to determine the MBC of *P. rubra* flower extract for different bacteria and under different optical fluences. To this end, bacterial inocula were prepared in advance to provide an aqueous solution containing 10^5 CFU/mL.

Using a 96-well flat-bottom plate, 150 μL of sterile water was added to the wells of rows B through H and columns 1 through 3 (in triplicate). Then, 150 μL of an aqueous solution containing 50.00 mg/mL of *P. rubra* extract was added to the wells of rows A and B. A serial dilution was then performed by transferring 150 μL from the wells of row B to the wells of row C, and this process was repeated successively up to row G. The wells of row H were kept free of extract to verify the effect of light on bacterial multiplication.

Continuing the assay, 15 μL of the bacterial inoculum was added, resulting in approximately 10^5 CFU/mL in each well. After inoculation, the 96-well plate was kept in the dark for 15 min to allow the extract to interact with the bacteria.

Next, to determine the effect of the extract in the absence of light, 3 μL from each well was transferred to a Petri dish containing solid medium (PCA agar) using an eight-channel

micropipette to obtain three vertical lines with eight pipetting points. An arrow was drawn on the bottom of the Petri dish to indicate the direction of increasing extract concentration. The plate was kept in a laminar flow hood to dry and then transferred to incubation at 37 °C for 24 h.

To evaluate the effect of the extract on bacteria when exposed to light, i.e., photodynamic inactivation, the same 96-well plate was subjected to illumination using the previously described light source for 15 minutes. After this illumination period, 3 μL from each well was transferred to a new Petri dish containing solid medium, using an eight-channel micropipette, to obtain three vertical lines with eight pipetting points each. An arrow was drawn at the bottom of the Petri dish to indicate the direction of increasing extract concentration. The Petri dish was kept in a laminar flow chamber for drying and then incubated at 37 °C for 24 h.

The same plate was illuminated again for the same time interval. The process of transferring 3 μL to a Petri dish, drying, and incubation was repeated, resulting in 4 Petri dishes associated with exposure times of 0, 15, 30, and 45 min, corresponding to optical fluence values of 0, 9, 18, and 27 J/cm^2 . The entire process described above was repeated for each bacterium separately.

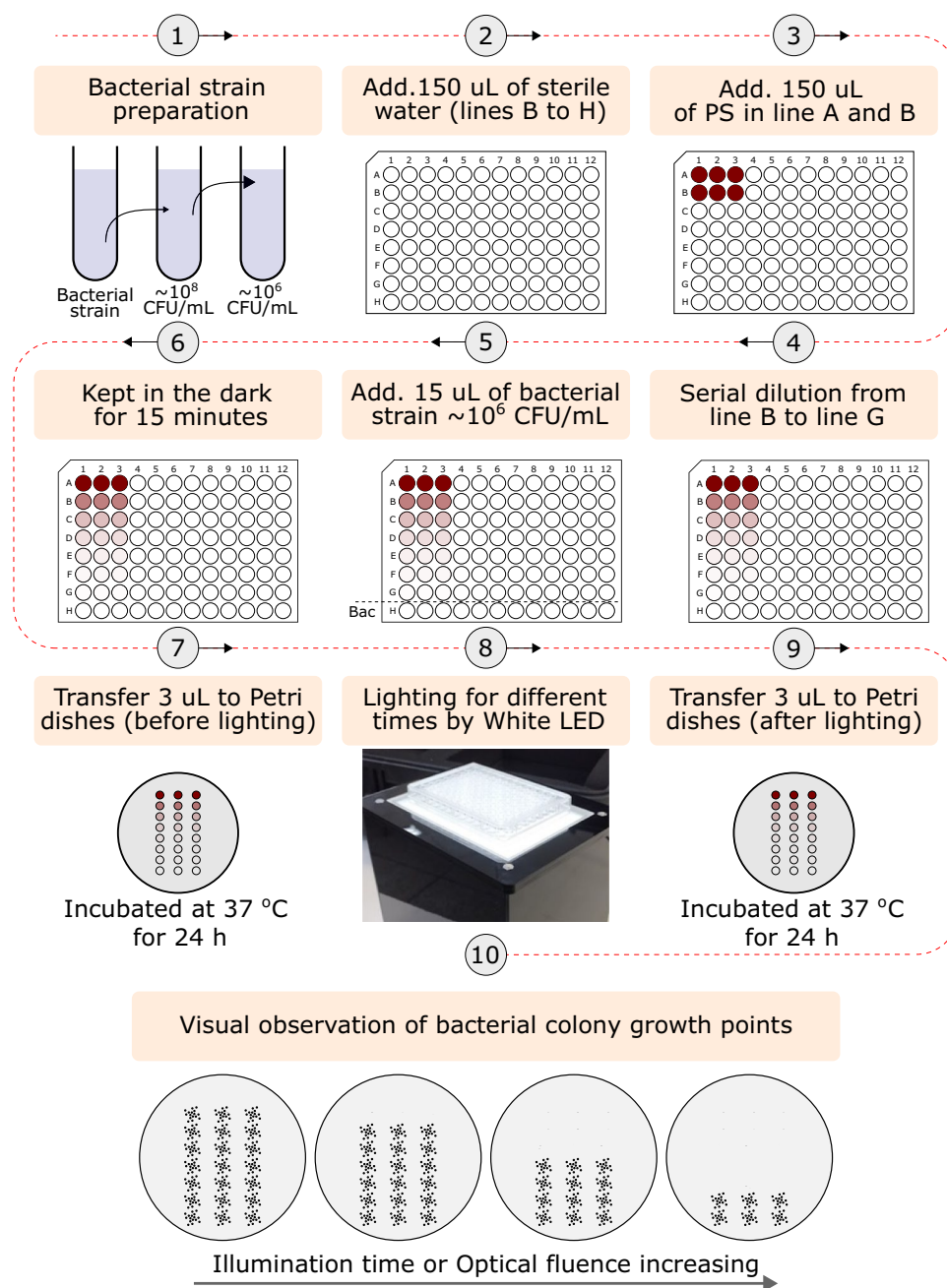
After the Petri dish incubation period, the Petri dishes were photographed, and their images were organized and cropped to show the area of interest (inside the dish) using the vector drawing software Inkscape.

The MBC was determined by identifying the pipetting point on each dish corresponding to the lowest extract concentration that showed no visible bacterial growth. Figure 3 presents a graphical description of the procedure adopted for the aPDI assay.

Results and discussion

As shown in Table 1, the extract of *P. rubra* flowers presents phenolic compounds divided into five classes. The flavonol class was predominant, with rutin (quercetin-3-*O*-rutinoside) being the primary compound, with a concentration of 83.96 mg/g, followed by kaempferol-*O*-pentoside-hexoside (4.04 mg/g) and quercetin-3-*O*-glucoside (2.90 mg/g). Among the hydroxycinnamic acids, chlorogenic acid stood out as the main compound, with 13.38 mg/g, while p-coumaric acid glucoside presented 0.59 mg/g. In the iridoid class, α -protoplumericin B/C was the most abundant (2.89 mg/g), while caffeoyl-15-demethyl-plumieride was detected in smaller amounts (0.07 mg/g). Regarding phenylpropanoids, only cerberic acid B was identified, at a concentration of 2.95 mg/g. Additionally, in the hydroxybenzoic acid class,

Fig. 3 Schematic of the aPDI assay: (1) inoculum preparation; (2–6) microplate preparation; (7) dark control (transfer of 3 μ L before illumination); (8) light exposure; (9) transfer of 3 μ L after illumination; (10) assessment of the absence or presence of bacterial growth



3,4-dihydroxybenzoic acid was present at a concentration of 0.12 mg/g.

The *P. rubra* flowers extract stands out for presenting rutin at a higher concentration. Rutin is considered a potent free radical inhibitor and scavenger, capable of protecting lipids from peroxidation and modulating inflammatory pathways. Its effects include cardiovascular, neuroprotective, antidiabetic, analgesic, gastroprotective, anticancer, and dermatological protection, as well as antiviral, antibacterial, and antispasmodic activities (Casa et al. 2000; Yang et al. 2008; Mittal et al. 2018; Iriti et al. 2023; Bazayr et al. 2023; Forouzanfar et al. 2025).

Studies have shown that the combination of rutin and PS can significantly improve the efficacy of aPDT. This improvement stems from the presence of saccharide groups in rutin, which act to orient PS toward the polysaccharide domains of bacterial cell walls, facilitating PS permeation. Furthermore, in a study using Methylene Blue (MB) as PS, the interaction with rutin alters MB from its dimeric form to the monomeric form, which is more active for aPDT. Thus, rutin acts on both the aPDT process and the chemical stability of PS (Motallebi et al. 2020; Szpakowska et al. 2001).

Figure 4 shows the results from the antioxidant activity assay (TBARS) by the absorbance values at 532 nm as a

Table 1 Tentative identification and quantification of phenolic compounds from *P. rubra* flower extract

Peak	Rt (min)	λ_{m} (nm)	[M–H] [–] (m/z)	MS ² (m/z)	Molecular formula	Assignment	Class	Con- cen- tration (mg/g)
1	5.37	nd	325.09299	163.04005, 119.05020	C ₁₅ H ₁₈ O ₈	<i>p</i> -Coumaric acid glucoside	Hydroxycinnamic acid	0.59 ± 0.01
2	6.30	nd	153.0194	109.02953	C ₇ H ₆ O ₄	3,4-Dihydroxybenzoic acid	Hydroxybenzoic acid	0.12 ± 0.01
3	7.63	324	353.08755	191.05614, 179.03532	C ₁₆ H ₁₈ O ₉	Chlorogenic acid	Hydroxycinnamic acid	13.38 ± 0.46
4	10.95	280	209.04552	191.03503	C ₁₁ H ₁₀ O ₅	Cerberic acid B	Phenylpropanoid	2.95 ± 0.06
5	13.82	256, 352	741.1882	300.02753, 301.03561	C ₃₂ H ₃₈ O ₂₀	Quercetin- <i>o</i> -rhamnoside-pentoside-hexoside	Flavonol	2.23 ± 0.10
6	15.36	352, 256	595.13037	300.02756, 301.03275	C ₂₆ H ₂₈ O ₁₆	Quercetin- <i>o</i> -pentoside-hexoside	Flavonol	0.67 ± 0.05
7	15.49	356, 256	595.13019	300.02756, 301.03537	C ₂₆ H ₂₈ O ₁₆	Quercetin- <i>o</i> -pentoside-hexoside	Flavonol	2.12 ± 0.04
8	16.82	356, 256	609.14563	300.02750, 301.03525	C ₂₇ H ₃₀ O ₁₆	Quercetin- <i>o</i> -neohesperidoside	Flavonol	2.27 ± 0.15
9	17.08	256, 356	609.14563	301.03247, 300.02750	C ₂₇ H ₃₀ O ₁₆	Rutin	Flavonol	83.96 ± 1.99
10	17.61	256, 356	739.1733	300.02753, 595.13037, 637.14105, 677.17279	C ₃₂ H ₃₆ O ₂₀	Quercetin- <i>o</i> -hexoside-pentoside-methylglutarate	Flavonol	0.18 ± 0.01
11	18.62	256, 352	463.0000	301.02753	C ₂₁ H ₂₀ O ₁₂	Quercetin-3- <i>o</i> -glucoside	Flavonol	2.90 ± 0.02
12	18.63	256, 353	579.135	284.03247, 285.04034	C ₂₆ H ₂₈ O ₁₅	Kaempferol- <i>o</i> -pentoside-hexoside	Flavonol	4.04 ± 0.03
13	19.02	344, 264	593.15143	284.03565, 285.04047	C ₂₇ H ₃₀ O ₁₅	Kaempferol-3- <i>o</i> -rutinoside	Flavonol	0.08 ± 0.20
14	18.89	296, 320	779.2043	179.03494, 617.15106, 213.05566, 573.16132	C ₃₅ H ₄₀ O ₂₀	Caffeoyl-15-demethyl-plumieride	Iridoid	0.07 ± 0.01
15	19.59	304	763.2088	213.05573, 169.06587	C ₃₅ H ₃₉ O ₁₉	α-Protoplumericin B or C	Iridoid	2.89 ± 0.02
16	19.93	292	763.20917	213.05566, 169.06586, 163.03999	C ₃₅ H ₃₉ O ₁₉	α-Protoplumericin B or C	Iridoid	0.20 ± 0.01
17	20.53	356, 256	607.13068	300.02753, 301.03531, 463.08804, 505.09875, 545.13043	C ₂₇ H ₂₈ O ₁₆	Quercetin- <i>o</i> -hexoside-dimethylglutarate	Flavonol	0.75 ± 0.04
18	20.92	344, 264	447.0344	285.04047, 284.03262	C ₂₁ H ₂₀ O ₁₁	Kaempferol- <i>o</i> -galactoside	Flavonol	0.09 ± 0.01
19	22.09	344, 264	447.0928	285.03650, 447.09665	C ₂₁ H ₂₀ O ₁₁	Kaempferol-3- <i>o</i> -glucoside	Flavonol	0.59 ± 0.01
20	24.75	348, 264	591.13562	285.04050, 447.09354, 489.10394, 529.13477	C ₂₇ H ₂₈ O ₁₅	Kaempferol-3- <i>o</i> -glucoside-6''-(3-hydroxy-3-methylglutarate)	Flavonol	0.60 ± 0.06
21	30.31	312	601.15631	169.06590, 213.05574, 163.04007	C ₂₉ H ₃₀ O ₁₄	15-demethyl-plumieride- <i>p</i> - <i>E</i> -coumarate	Iridoid	0.13 ± 0.01

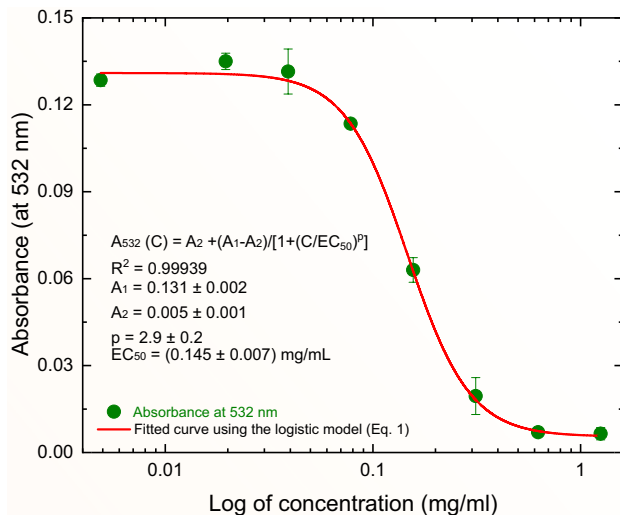


Fig. 4 Absorbance at 532 nm as a function of the concentration of *P. rubra* flower extract, subjected to the TBARS assay to evaluate antioxidant activity. The EC_{50} value was obtained by theoretical fitting using a logistic model (Eq. 1). The parameter p reflects the sensitivity of the extract's antioxidant activity to variations in concentration

function of the concentration of the extract from the flowers of *P. rubra* together with the theoretical adjustment (red line) using a logistic model given by Eq. 1, which allowed obtaining the $EC_{50} = (0.145 \pm 0.007)$ mg/mL and a sensitivity parameter $p = 2.9 \pm 0.2$

For $p = 1$, a typical sigmoid response is observed, in which the antioxidant effect increases progressively and continuously with extract concentration. When the p parameter assumes values less than 1 ($p < 1$), the relationship between absorbance and extract concentration presents a more gradual transition, indicating that increasing concentration promotes a less uniform response. This behavior may suggest the presence of multiple antioxidant compounds with different reducing potentials or low homogeneity in the antioxidant process (Sorribas et al. 2007; Noel et al. 2018).

On the other hand, $p > 1$ indicates a more abrupt transition, in which slight variations in concentration around the EC_{50} result in significant changes in antioxidant activity. This behavior suggests the occurrence of cooperative or synergistic mechanisms between the antioxidant compounds present in the extract, which may be associated with the *P. rubra* extract, given the observed value for the p parameter of 2.9 ± 0.2 (Sorribas et al. 2007; Noel et al. 2018).

Figure 5 shows the absorbance spectra of *P. rubra* flower extracts at different concentrations, ranging from 30.00 to 0.23 mg/mL. The extract exhibits a broad optical absorption range, extending from approximately 400 to 630 nm. Based on this spectral behavior, we chose to use a light source composed of a set of LEDs emitting white light, characterized by two broad bands centered around 450 and 550 nm, in the aPDI assays.

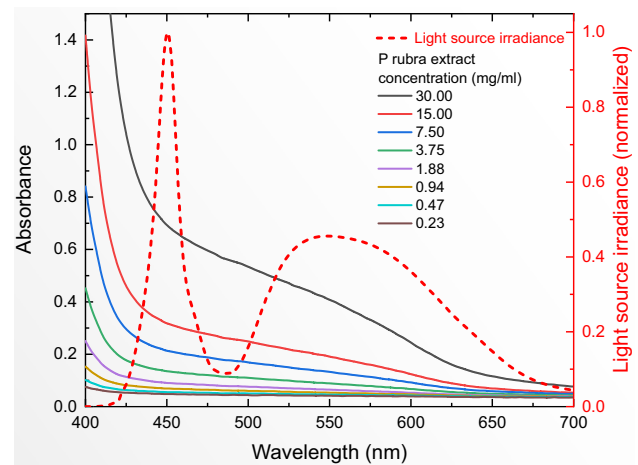


Fig. 5 UV-VIS spectra of *P. rubra* flower extract in aqueous solution under different concentrations (solid lines) and irradiance of the light source used in the aPDI assay (red dashed line)

The choice of white light, in addition to presenting an adequate spectral match with the extract's absorption region, offers practical and economic advantages, since such sources are widely commercially available, low-cost, and do not require the use of lasers and optical filters, characteristics that make them particularly attractive for industrial-scale applications.

The antimicrobial activity assay, in the dark, was performed to verify whether the extract exhibits antimicrobial activity (in the dark) and at what MIC and MBC values this occurs. The results show that, within the investigated concentration range of 0.1 to 100.00 mg/mL, the extract did not exhibit antimicrobial activity. That is, both MIC and MBC were considered greater than 100 mg/mL indicating that the *P. rubra* extract does not exhibit antimicrobial activity in the dark, which is a desirable characteristic in a PS. Based on this result, aPDI assays were performed to investigate different concentrations of the extract obtained by serial dilution, starting from 50.00 mg/mL.

The methodology used in the aPDI assay did not aim to quantify microbial reduction, but rather to determine the minimum extract concentration capable of exhibiting bactericidal action, as well as the corresponding optical fluence values. To this end, the images of the Petri dishes were analyzed to identify the pipetting points that showed no visible signs of microbial growth.

Figure 6 shows the images of the Petri dishes for the different bacterial strains, at the four light doses used: 0 J/cm² (dark control), 9 J/cm², 18 J/cm², and 27 J/cm², along with the respective extract concentrations corresponding to each pipetting point.

During the analysis of the Petri dish images, care was taken to distinguish between the marks left by the micropipette tip's contact with the solid medium and possible

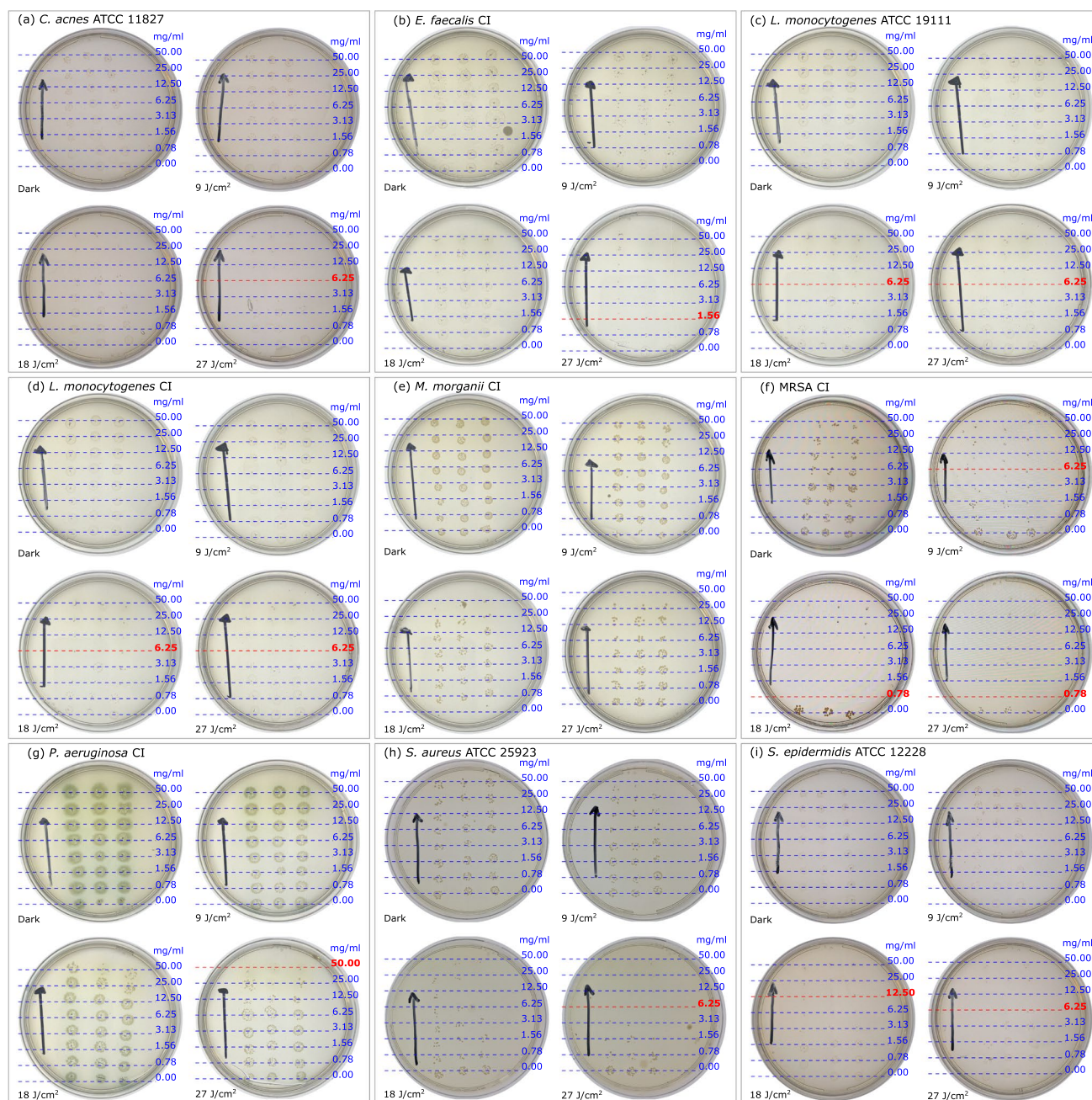


Fig. 6 Photographs of Petri dishes for different bacterial strains and optical fluence values (Dark = 0 J/cm²). The arrow drawn on the background of each plate indicates the direction of increasing *P. rubra* extract concentration, ranging from 0 to 50.00 mg/mL

signs of bacterial growth. The distinction between these two types of marks is easily discernible: while the tip's contact produces a well-defined and localized spot, bacterial growth manifests itself as a diffuse halo, with a larger area and irregular distribution. Therefore, only pipetting points that did not show any visible evidence of bacterial colonies were considered free of multiplication and used for the determination of MBC.

In the aPDI assay, including the dark control, it was observed that the *P. rubra* flower extract showed no

antimicrobial activity at concentrations up to 50.00 mg/mL. This result was expected, since similar behavior had been observed in the antimicrobial activity assay performed in the absence of light.

When applied at an optical fluence of 9 J/cm², the extract was found to inactivate only the MRSA CI strain, presenting a MBC of 6.25 mg/mL. At an optical fluence of 18 J/cm², the MBC for MRSA CI decreased to 0.78 mg/mL, while the MBC for *L. monocytogenes* (ATCC and CI) were 6.25 mg/mL, and for *S. epidermidis*, 12.50 mg/mL.

Under an optical fluence of 27 J/cm^2 , inactivation of all bacterial strains tested was observed, except for *M. morgani*. Under these conditions, the MBC was 6.25 mg/mL for *C. acnes*, *L. monocytogenes* (ATCC and CI), *S. aureus*, and *S. epidermidis*; 1.56 mg/mL for *E. faecalis*; 0.78 mg/mL for MRSA CI; and 50.00 mg/mL for *P. aeruginosa*.

Figure 7 presents a comparative graph of the MBC for each bacterial strain under different optical fluence regimes, as determined from the analysis of Petri dish images.

The results obtained in the aPDI assay demonstrated that *P. rubra* extract exhibits photodynamic inactivation effects against various bacteria. The exceptions were *M. morgani*, which was not inactivated under any of the tested conditions, and *P. aeruginosa*, which was inactivated only at the highest optical fluence and extract concentration. This is because, unlike the others, the two bacteria are Gram-negative characterized by a dual-membrane cell wall structure which makes them more resistant to aPDI than Gram-positive bacteria (Rapacka-Zdonczyk et al. 2021).

Conclusion

The extract from the flowers of *P. rubra* is a rich source of phenolic compounds, with rutin being the main constituent (83.96 mg/g). This phytochemical composition confers the

extract a high antioxidant potential, evidenced by the cooperative behavior between its components ($p = 2.9$).

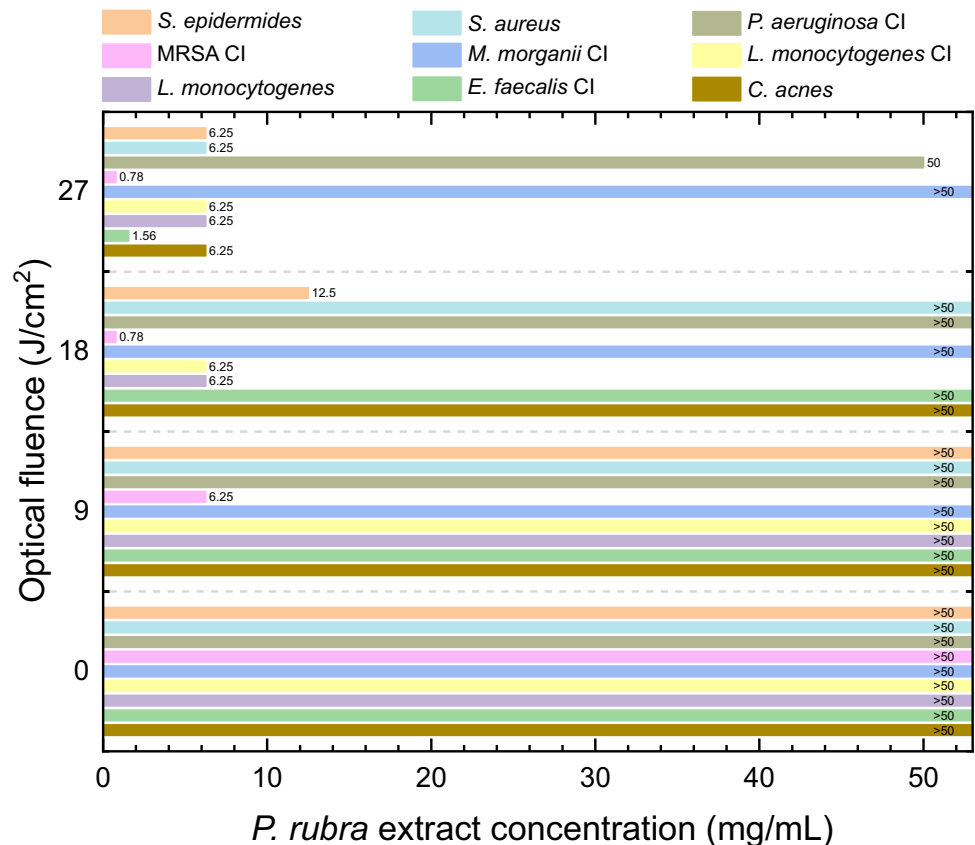
The absence of antimicrobial activity in the dark (MIC/MBC $> 100 \text{ mg/mL}$) is a crucial characteristic for a PS. When exposed to white light (an affordable and accessible source of irradiation), the extract exhibited significant aPDI against Gram-positive bacteria, including multidrug-resistant strains (MRSA CI, MBC = 0.78 mg/mL at 18 J/cm^2). The efficacy was fluence-dependent, inactivating most strains at 27 J/cm^2 , except the most resistant Gram-negative strains.

In summary, the extract from the flowers of *P. rubra* is a promising natural PS, combining a superior phytochemical profile, inactivity in the dark, and potent photodynamic bactericidal action, validating its potential for applications in aPDI.

Limitations and future perspectives

Future studies should include in vivo models to validate the efficacy observed under in vitro conditions and broaden the biological relevance of the results. The incorporation of three-dimensional models and biofilm-based systems is recommended, as they more accurately reflect microbial organization and tolerance mechanisms associated with

Fig. 7 MBC of *P. rubra* flower extract as a function of the optical fluence applied to different bacterial strains. Values greater than 50 mg/mL indicate that the extract did not present aPDI within the range of concentrations and optical fluences tested



persistent infections. Additionally, future studies should standardize the process for obtaining *P. rubra* extract and systematically evaluating the influence of environmental factors related to the region and cultivation conditions, such as climate, edaphoclimatic characteristics, and plant development stage, on its photodynamic activity. Finally, expanding the number and diversity of microbial strains evaluated will allow for a more comprehensive understanding of photodynamic efficacy in the face of genetic and phenotypic heterogeneity and distinct resistance mechanisms of clinical relevance.

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Data Availability No datasets were generated or analysed during the current study.

Declarations

Competing interests The authors declare no competing interests.

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