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Antimicrobial aqueous aerosol formulation using a propolis extract from eutectic solvent extraction

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ABSTRACT

Propolis, naturally produced by honeybees for the construction and protection of beehives, exhibits numerous health benefits, particularly antimicrobial activity derived from phenolic compounds abundant in Brazilian Green propolis. However, conventional extraction methods and solvents can be environmentally hazardous and potentially harmful to skin, evidencing the need to develop safe solutions without compromising antimicrobial effects, such as the use of eutectic solvent (ES).Although the use of ES for the extraction of phenolic compounds and the antimicrobial activity of propolis are well established, this work is the first to investigate propolis as an active ingredient in the formulation of aqueous-based antiseptic aerosols. A propolis-based extract was developed using ultrasound-assisted extraction (UAE) and a novel ES composed of betaine and 1,4-butanediol in a 1:1 M ratio, and a solid-liquid ratio of 0.02 $g_{propolis}.mL_{solvent}^{-1}$. This methodology yielded an extract with a total phenolic content of 139 ± 15 $mg_{GAE}.g_{propolis}^{-1}$, effectively inactivating methicillin-resistant *Staphylococcus aureus* (MRSA) and *Pseudomonas aeruginosa* with minimum inhibitory concentration (MIC) values of 0.174 $mg_{GAE}.mL_{solvent}^{-1}$ and 0.696 $mg_{GAE}.mL_{solvent}^{-1}$, respectively. Furthermore, an aerosol prototype for skin application was successfully developed using this extract. *Ex vivo* assays on porcine skin confirmed its efficacy, showing a 2-log inactivation of MRSA after 24 h. Cell viability assays in human keratinocytes indicated the biocompatibility of the formulation with the skin. These findings serve as a proof of concept, showing the effectiveness of propolis formulation against bacteria and its potential application as an aqueous aerosol for treating skin infections.

1. Introduction

Infections have been an enduring threat to global public health [1]. In 2019, 13.7 million infection-related deaths were reported. This scenario has been aggravated by the rise of antimicrobial resistance (AMR), largely caused by the misuse and overuse of antibiotics. AMR-related deaths are predicted to reach one every 3 s by 2050 [1,2], highlighting the urgent need for new and sustainable alternatives [3].

Propolis is a natural resinous material gathered by bees from plant buds and exudates mixed with the bee's salivary and enzymatic secretions, essential for building and protecting the hives [4]. Different types of propolis can be found in nature, with their chemical composition determined mainly by factors such as geographical location, soil characteristics, bee species, and local flora [4]. More than 850 compounds have been reported in samples worldwide, particularly phenols, flavonoids, terpenes, steroids, esters, vitamins, and minerals [4,5].

Propolis exhibits strong antimicrobial activity and has been evaluated against over 600 bacterial strains, including antimicrobial-resistant

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ones [5]. It acts by stimulating the host's immune system or directly interacting with the pathogen [4,5]. This multi-target mode of action and natural variability diminishes the likelihood of bacterial resistance [5]. Green propolis, primarily sourced from *Baccharis dracunculifolia* in Brazil, is distinguished by its high content of baccharin, *p*-coumaric acid, and artepillin C, compounds of significant interest to medical and pharmaceutical industries considering their reported antimicrobial, antioxidant and anti-inflammatory properties [6,7]. Thus, extracting its bioactive compounds is vital to fully harnessing its properties. This process removes waxes and debris while increasing the solubility of the desired bioactive compounds [8]. Solid-liquid extraction methods, such as ultrasound-assisted extraction (UAE), are commonly used for this purpose [8]. The UAE has proven to be an efficient technique, as it works primarily through cavitation [8,9]. This technology improves the solubility of the propolis bioactive compounds in the solvent, and offers, when properly optimized, high yields, short extraction times, and compatibility with various solvents [8,9].

Choosing the appropriate solvent is critical, as its properties directly influence extraction yield, selectivity, and key formulation characteristics, namely, viscosity, antioxidant activity, and biological performance [3,8]. Ethanol is the most used solvent for extracting phenolics from propolis due to its high affinity for propolis compounds and its widespread availability [3,8]. However, ethanol may not be appropriate for certain groups, such as pregnant women and children, or for specific industries, including cosmetics and pharmaceuticals [8,10]. Water is also used as an extraction solvent, solving some of ethanol's limitations, although it may be less efficient for certain biomolecules, due to its low selectivity, reduced extraction yields, and diminished biological activity [4,8].

Several studies suggest eutectic solvents (ES) as promising candidates to replace and overcome the most common drawbacks associated with more conventional solvents [11–14]. These solvents are formed by combining hydrogen bond acceptors (HBA) with one or more hydrogen bond donors (HBD) through simple methods, resulting in highly versatile and task-specific solvents [11,15]. Their high solubilization power enables the extraction of compounds with poor water solubility, allowing for increased water content while maintaining high extraction yields [12,15,16]. Various HBA:HBD combinations have demonstrated high extraction efficiency for phenolic compounds from propolis [16,17]. Examples from the literature using UAE include L-proline:D,L-lactic acid:water in a 1:2:2.5 M ratio [18], betaine:citric acid in a 1:2 M ratio [19], and citric acid:1,2-propanediol in a molar ratio 1:4 [20]. Additionally, certain ES formulations exhibit intrinsic antimicrobial properties, which could enhance the antimicrobial activity of the extracted bioactive compounds through a synergistic effect [11,21]. These features highlight the potential of ES as extraction media for propolis, acting as carriers for the phenolic compounds responsible for its antimicrobial activity and providing a promising platform for fighting infections.

The skin is a primary interface with the external environment, and many pathogens enter our organism through contact with infected surfaces or by directly infecting underlying soft tissues [22,23]. This can lead to systemic dissemination and sepsis, particularly when the skin barrier is compromised [22,23]. Therefore, effective infection control relies on safe and efficient disinfection of the skin [23,24].

In this context, antiseptic and antimicrobial aerosols are commonly used due to their practicality for both hygiene and treatment purposes [24,25]. These sanitizers are typically ethanol-based due to their ability to rapidly and effectively eliminate microorganisms [26]. However, prolonged use can be detrimental to the skin, causing pain (especially in the presence of wounds), irritation, contact dermatitis, and overall disruption of skin integrity [10,26]. Moreover, ethanol's high volatility can lead to unintentional inhalation of vapors and reduce its lasting effectiveness [27,28]. This underscores the need for safer aerosol formulations that remain effective against pathogens [23,27,28].

Although the use of ES for the extraction of phenolic compounds and the antimicrobial activity of propolis are well established, to date no studies have explored propolis as an active ingredient in antiseptic aqueous aerosols. Propolis-based aerosols are primarily oral or throat sprays, often intended for dental applications, and all formulated using water, ethanol, or mixtures of both [29]. Therefore, incorporating propolis with an ES in an antiseptic and/or antimicrobial aerosol for skin disinfection represents a novel approach [30]. Additionally, the ES formulation could be skin-safe and non-volatile, allowing for prolonged application on infected areas [11,31].

Considering these challenges, this study aims to develop an aqueous antimicrobial aerosol for skin disinfection. This was achieved by extracting propolis' phenolic content using ES combined with UAE, to obtain a high yield extract with antibacterial properties. The formulated aerosol was then evaluated *in vitro* and *ex vivo*, to serve as proof of concept for its effectiveness as a skin disinfectant.

2. Materials and methods

2.1. Materials

Ethanol absolute (HPLC grade, CAS 64–17-5) was obtained from Fisher Scientific. The ES were prepared using betaine anhydrous (98 wt %, CAS 107–43-7, Thermoscientific), 1,4-butanediol (99 wt%, CAS 110–63-4, Thermoscientific), and distilled water. A Folin-Ciocalteu assay was performed, using Folin-Ciocalteu reagent (CAS 12111–13-6, Panreac), gallic acid (99.5 wt%, CAS 5995-86-8, Merck), and sodium carbonate (99.9 wt%, CAS 497–19-8, Prolabo). For the cell cytotoxicity studies, trypsin, penicillin–streptomycin solution, fetal bovine serum, dimethyl sulfoxide, and thiazolyl blue tetrazolium bromide (MTT) were acquired from Sigma–Aldrich (Merck KGaA), and Dulbecco's modified Eagle's medium with high glucose (DMEM) was purchased by Biowest (Nuaillé). Raw green propolis was sourced from Brazil and generously donated by Mn Propolis. Tryptic Soy Broth (TSB) and Tryptic Soy Agar (TSA) were obtained from Liofilchem.

2.2. Propolis extract preparation

Frozen raw Brazilian green propolis samples were ground using a domestic blender (Moulinex, 1000 W) and sieved through a steel mesh to obtain particles ranging from 0.5 to 1 mm in size. The processed samples were then stored at −18 °C until further use.

The ES was prepared using a method adapted from Abbot et al. [32], at a 1:1 M ratio, with betaine as HBA and 1,4-butanediol as HBD, and 50 wt% of water content (Bet:But 1:1). The predetermined amounts of the starting materials were placed in a sealed glass flask and stirred overnight at room temperature (25 °C) until a homogeneous, transparent liquid was obtained.

The extraction of propolis' compounds was adapted from the ultrasound-assisted extraction method optimized by Contieri et al. [19], using the ES and ethanol (as a control) as solvents. The extraction was performed with a solid-liquid ratio (SLR) of 0.02 g of propolis *per* mL of solvent, using an ultrasonic probe (SONICS VibraCell VCX 130, 130 W, 20 kHz) with 6 min of contact between propolis and the solvent, followed by 5 min of extraction, at room temperature. After, 2 mL aliquots were centrifuged at 15000 rpm for 15 min (Hettich MIKRO 200), and the supernatant was collected and stored at 4 °C until further use.

The pH of extracts was measured using a digital benchtop pH meter (METTLER TOLEDO SevenExcellence), and the viscosity of the extracts was assessed at 25 °C, 37 °C and 42 °C, using a Kinexus rheometer, and the rSpace for Kinexus software, both from Netzsch.

2.3. Quantification of the extracts' total phenolic content

The Total Phenolic Content of the propolis extracts was determined using the Folin-Ciocalteu colorimetric method, adapted from Coscueta et al. [33]. Before analysis, the extracts were diluted 1:6 in ethanol. The

assay was conducted in a 96-well microplate, where 20 μL of each diluted extract was mixed with 80 μL of the Folin-Ciocalteu reagent and 100 μL of anhydrous sodium carbonate solution (7.4% *w/v*) in duplicate. After a 30-min incubation at room temperature, absorbance was measured by UV–Vis spectroscopy at 765 nm using a microplate reader (BioTek Synergy HTX) operated with the Gen5 2.04 software. The Total Phenolic Content of the extracts was expressed as gallic acid equivalents (GAE), calculated using a gallic acid calibration curve (0.015–0.225 mg. mL^{-1}) prepared for each assay. The results were reported as mg of GAE *per* g of propolis.

2.4. Antibacterial assays

Bacterial Strains and Growth Conditions: The bacterial strains used in this study were a methicillin-resistant *Staphylococcus aureus* strain (MRSA DSM 25693), which produces staphylococcal enterotoxins A, C H, G, and I, [34] obtained from DSMZ – German Collection of Microorganisms and Cell Cultures GmbH, and a *Pseudomonas aeruginosa* strain, provided by Centro Hospitalar Universitário de Coimbra (CHUC), isolated from a patient with a respiratory illness. Both bacterial strains were cultured on solid TSA medium for 24 h at 37 °C, and subsequently stored at 4 °C. Before each assay, a single isolated colony was inoculated in 30 mL of TSB and grown aerobically under continuous stirring at 120 rpm and 37 °C for 18 to 24 h. Then, a 300 μL aliquot of this culture was transferred into fresh TSB liquid medium and incubated under the same growth conditions until it reached the stationary phase, at which point it was used for the assay.

Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC): Minimal Inhibitory Concentration (MIC) and Minimum Bactericidal Concentrations (MBC) of the extract and its respective solvent against MRSA and *P. aeruginosa* were determined using a microdilution method in a 96-well flat-bottomed microplate. Each assay included bacterial control wells (containing only the bacterial suspension), extract control wells, and ES control wells. Additionally, a control series containing extract and TSB medium was included to account for the extract's coloration. Each sample contained 100 μL of a bacterial suspension at the concentration of 1×10^6 colony-forming units *per* milliliter (CFU mL^{-1}) and 100 μL of propolis extract or solvent in the first well. Serial dilutions of the controls, propolis extract, and solvent samples were performed by transferring 100 μL from one well to the next. The samples and controls were then incubated at 37 °C for 24 h. Following incubation, the optical density was measured at 600 nm in a microplate reader (Thermo Scientific Multiskan FC Microplate Photometer). Three independent experiments were conducted. The MIC value was defined as the lowest concentration of extract or solvent at which no visible bacterial growth was observed [35,36]. For MBC determination, samples from wells showing no visible growth were serially diluted in PBS. Two drops (10 μL) from each dilution were cultured on TSA using the drop plate method and incubated at 37 °C for 18 to 24 h. After incubation, bacterial colonies were counted. The MBC value was defined as the lowest concentration of extract or solvent with no bacterial growth observed [36].

Kill Curves: Based on the MIC and MBC results, kill curve assays were performed using the propolis extract at 1.39 $mg_{GAE}.mL^{-1}_{solvent}$ and the respective ES concentration [50% (v_{ES}/v_{total})] against both MRSA and *P. aeruginosa*. A bacterial suspension of approximately 1×10^6 CFU. mL^{-1} was used. The assays included test samples (bacteria plus extract and bacteria plus eutectic solvent) along with bacterial, extract, and solvent controls, all incubated under identical conditions at 37 °C for 24 h. Aliquots from test samples and controls were collected at 0, 2, 6, 12, and 24 h of incubation, except for solvent and extract controls, which were sampled only at 0 and 24 h to assess potential bacterial contamination that could interfere with the assays. Each collected sample was serially diluted in PBS, and the appropriate dilutions were cultured in duplicate on TSA medium using both the drop plate and pour plate methods. After 18 to 24 h of incubation at 37 °C, the resulting colonies were counted. The viable cell concentration was expressed as $\log_{10}$ CFU. mL^{-1}. Three independent experiments were performed.

Antibiogram: The antibiogram was performed using the disc diffusion method with the propolis extract and respective solvent. The optical density of the MRSA bacterial culture was adjusted to 0.08 of OD600, corresponding to a bacterial concentration of 1×10^8 CFU mL^{-1}. The bacterial culture was evenly spread over the surface of solidified TSA plates using a sterile swab spreader. Discs loaded with 20 μL of the propolis extract (at the original concentration of 2.79 $mg_{GAE}.mL^{-1}_{solvent}$) and the respective ES concentration [100% (v_{ES}/v_{total})], were placed on the inoculated TSA plates, alongside the antibiotic piperacillin (30.0 μg, positive control) and a blank disc (negative control). The plates were then incubated for 24 h at 37 °C. The diameter of the inhibition zone surrounding the discs was measured in cm.

2.5. Aerosol demonstrator

The aerosol was prepared in collaboration with Colep Consumer Products Portugal, S.A., located in Vale de Cambra, Portugal (https://colep-cp.com). The aerosol consists of a standard aluminum can, equipped with an actuator like a deodorant, loaded with 40% of compressed N_2 gas and 60% of undiluted Bet:But extract. Before filling the aerosol can, the extract samples were filtered using a vacuum pump to remove any debris or impurities that could interfere with the aerosol's performance. Additionally, an aerosol containing only the Bet:But 1:1 solvent was prepared under the same conditions and used as a control.

2.6. Antibacterial studies with the aerosol

Inactivation Assays on Agar Plates: To assess whether the aerosol retained the antibacterial properties of the extract, TSA plates inoculated with MRSA at a concentration of 1×10^8 CFU mL^{-1} were sprayed one, two, and three times (approximately 100 μL of solution *per* spray). The plates were then incubated at 37 °C for 24 h, after which the inhibition profile was observed.

Inactivation Assay on Porcine Skin: The evaluation of bacterial inactivation, using the propolis extract aerosol, in *ex vivo* models, was conducted following a protocol adapted from Braz et al. [37] Fresh porcine skin was obtained from a local butcher shop, then prepared and disinfected before contamination with MRSA. First, the adipose tissue beneath the dermis was removed, and the skin was cut into 4 cm^2 pieces (2×2 cm^2), which were placed in sterile petri dishes. To reduce the bacterial load already present on the skin, the excised skin pieces were disinfected by exposure to ultraviolet radiation (UV-C sterilizing chamber) [38] for 15 min on each side.

Bacterial Inactivation on porcine skin (*ex vivo* assays): After preparing the porcine skin, the excised and disinfected pieces were placed in 6-well plates for infection with MRSA and testing with Bet:But extract aerosol and ES Bet:But 1:1 aerosol. Bacterial control pieces were also included. An overnight culture of MRSA was diluted in TSB to a final concentration of 10^5 CFU mL^{-1}, and a 200 μL aliquot was applied to the skin pieces, which were left to incubate for 30 min. Then, two sprays of the respective aerosols (approximately 100 μL *per* spray) were applied to the skin pieces, while 200 μL of PBS was added to the bacterial control pieces to match the final volume (400 μL total). The samples were incubated for 15 min to allow the aerosols to interact with the infected skin. To maintain a moist environment for the skin samples, sterile PBS was dispensed between the plate wells before incubation at 25 °C for 24 h. At 0 and 24 h, samples were collected by adding 1 mL of PBS to each well, followed by aspiration and dispensing 10 times to promote bacterial detachment. The collected solution was then serially diluted in PBS. Bacterial concentration was determined in triplicate on TSA medium for each time point using the drop plate method, with incubation at 37 °C for 18 to 24 h. Three independent experiments, each with two replicates, were performed.

2.7. Cytotoxicity assays

Cytotoxicity studies were performed using the human keratinocytes cell line HaCaT. Cells were maintained in culture medium (DMEM with high glucose level supplemented with 10% fetal bovine serum and 1% penicillin–streptomycin), at 37 °C under a humidified air atmosphere containing 5% CO_2 in air. Approximately 6×10^3 cells were seeded *per* well in 96-well plates in 200 μL of culture medium and incubated for 24 h. Subsequently, HaCaT cells were exposed for a 24-h period to the solvent Bet:But 1:1 or to the extract samples at final concentrations ranging from 1 to 37.5 $\mu g.mL^{-1}$ and corresponding solvent dilutions. The MTT reduction assay was then carried out as previously reported. [39] The absorbance values for the untreated control cells were considered as 100% of cell viability. Three independent experiments were performed, and four replicate cultures were used in each experiment.

2.8. Statistical analysis

A one-way analysis of variance (ANOVA) was performed, followed by the Bonferroni post-hoc test to compare the results obtained in the studies conducted during this work. The results are presented as the mean ± standard error of the mean. Statistical significance was determined at 95% confidence level (p-value <0.05). All analyses were conducted using JAMOVI (Version 2.3) Software.

3. Results and discussion

3.1. Extraction and characterization of propolis active compounds

A schematic representation of the extraction process and aerosol demonstration is presented in Fig. 1.

To develop an efficient extraction process using ES, selecting the appropriate solvent is essential [19]. Betaine was chosen as HBA because it is a naturally occurring and safe compound found in plants and is widely used in the cosmetic and skincare industries due to its natural moisturizing properties [19,40] 1,4-butanediol was chosen as HBD for its compatibility with betaine, potential to develop a non-toxic formulation, and established safety profiles, with prior use in the pharmaceutical and cosmetic industries [41].

The ES was prepared at a 1:1 M ratio, with 50 wt% of water content, ensuring an aqueous-based formulation. Ethanol was evaluated as a control solvent due to its common use in literature, known for typically providing superior yields [4,19]. Upon completing the extractions, the yield of each solvent was evaluated by measuring the total phenolic content, expressed as gallic acid equivalents (GAE) *per* gram of propolis (Fig. 2A). Additionally, the extracts' pH was measured to evaluate their suitability for dermatological applications (Fig. 2A).

As shown in Fig. 2A, the Bet:But 1:1 ES, yielded 139 ± 15 $mg_{GAE}.g^{-1}_{propolis}$, equivalent to a concentration of 2.79 ± 0.30 $mg_{GAE}.mL^{-1}_{solvent}$, while ethanol, yielded 150 ± 1 $mg_{GAE}.g^{-1}_{propolis}$, equivalent to 3.01 ± 0.02 $mg_{GAE}.mL^{-1}_{solvent}$. This similarity in extraction yields indicates that the Bet:But ES is a viable alternative to ethanol, one of the most efficient solvents for propolis extraction, with no statistically significant

Fig. 1. Propolis extract preparation procedure: A) Ultrasound-assisted extraction of propolis extracts, and B) Aerosol production and bacterial activity assessment.

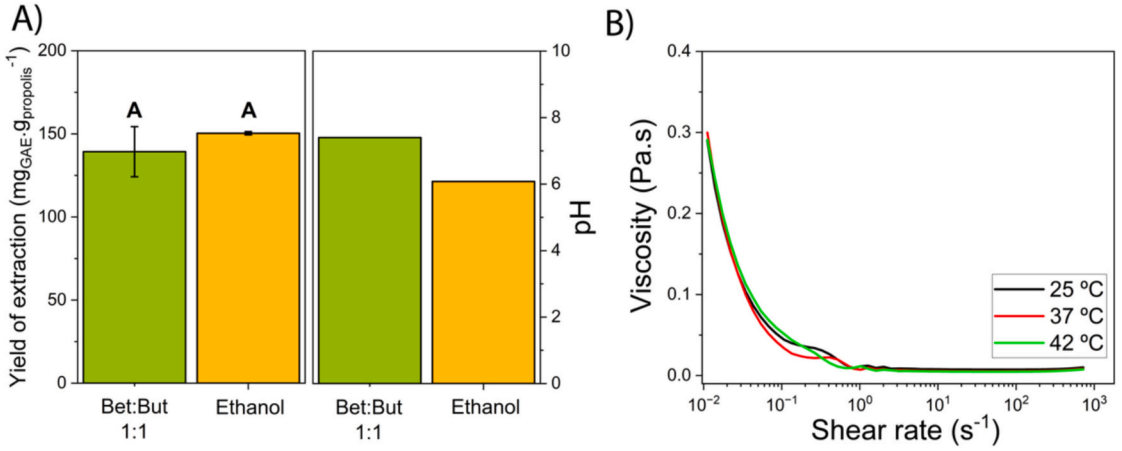

Fig. 2. Propolis extract characterization: A) Extraction yield of polyphenols ($mg_{GAE}.g_{propolis}^{-1}$) from green propolis using the Bet:But 1:1 ES, and ethanol absolute as a control, determined by the Folin-Ciocalteu colorimetric method. Different capital letters represent statistically different values in the conditions under study (p-value <0.05); B) Viscosity (Pa.s) *vs.* Shear Rate (s^{-1}) of Bet:But extract recorded at different temperatures. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

differences found both systems (p-value >0.05) [4,19]. Additionally, both tested solvents showed pH values within the natural pH range of skin (typically between 4 and 7), ensuring their suitability and safety for dermatological applications [42]. In particular, the Bet:But ES showed a pH of 7.40, which is also consistent with literature [42].

Although less frequently discussed in literature when compared to betaine, 1,4-butanediol is also recognized as a safe and sustainable ingredient [31,40]. It has applications in the medical and cosmetic industries, being reported for medical use, as well as in cosmetics [41,43,44]. Additionally, it is listed as an approved solvent in the International Cosmetic Ingredient Dictionary and Handbook [41,44]. Importantly, no adverse effects have been reported following topical application, supporting its suitability for dermatological use [41,44].

Based on both literature data and the results obtained in this study, the Bet:But solvent, at a molar ratio of 1:1 and with 50 wt% water, was selected for the extraction of polyphenolic compounds from green propolis and for subsequent use in the aerosol formulation.

To evaluate the suitability of the Bet:But extract for aerosol formulation, its viscosity was measured at three different temperatures: room temperature (25 °C), body temperature (37 °C), and 42 °C (Fig. 2B). The ES-based extract exhibited a pseudoplastic behavior, with dynamic viscosity decreasing as shear rate increased. This behavior was consistent across all tested temperatures. Furthermore, the viscosity of the formulation remained within the optimal range of aerosol applications at all shear rates [45]. These results indicate that, from a rheological standpoint, the Bet:But extract is well suited for use in a water-based aerosol formulation.

3.2. Antibacterial activity

After selecting the most appropriate solvent for extracting phenolic compounds from green propolis, it was essential to assess its antimicrobial properties to ensure that they retained their effectiveness post-extraction. While propolis has demonstrated antimicrobial activity against a wide range of microorganisms [5,13,14], this study specifically focused on bacteria. This choice was driven by the fact that bacteria are major contributors to skin infections, and the increasing prevalence of antibiotic bacterial resistance makes this research particularly pertinent [2,23,46]. The selected bacterial strains were MRSA DSM 25693 and *P. aeruginosa*, representing Gram-positive and Gram-negative models, respectively, with MRSA being of particular concern due to its resistance to multiple antibiotics [22,23,46].

Determination of MIC and MBC: The MIC and MBC were determined for the selected bacterial strains not only to confirm the antibacterial activity of the extract but also to establish their optimal concentration for further studies. The Bet:But extract and the ES Bet:But 1:1 were tested alongside bacterial control wells. Following the microdilution protocol, the concentration of each sample in the respective microplate wells is presented in Table 1, with well number 1 being the only bacteria-free well. The resulting MIC and MBC values are presented in Table 2.

The findings summarized in Table 2 suggest that for MRSA, the MIC is 0.174 $mg_{GAE}.mL_{solvent}^{-1}$ for the Bet:But extract, while the MBC is 0.696 $mg_{GAE}.mL_{solvent}^{-1}$. In contrast, the ES exhibits a MIC of 50% (v_{ES}/v_{total}), equivalent to a Bet:But extract concentration of 1.39 $mg_{GAE}.mL_{solvent}^{-1}$ (as found in well number 2 – Table 1). However, the MBC could not be determined, as bacterial growth was observed in all wells. These results suggest that the antibacterial activity is primarily attributed to the phenolic content extracted from propolis, since the ES had minimal influence, although enough to aid in the bacterial inactivation.

For *P. aeruginosa*, the MIC of the Bet:But extract is 0.697 $mg_{GAE}.mL_{solvent}^{-1}$, with an MBC of 1.39 $mg_{GAE}.mL_{solvent}^{-1}$. The ES presented a MIC of 25% (v_{ES}/v_{total}), corresponding to a Bet:But extract concentration of 0.697 $mg_{GAE}.mL_{solvent}^{-1}$ (found in well number 3 – Table 1), while the MBC remained undetermined. This suggests that in this bacterium, the antibacterial effect is not solely attributed to the phenolic content of the extract but also involves the ES, as indicated by the matching MIC values despite differences in MBC results.

Although the MIC and MBC data may seem relatively high, they confirm that both bacteria are susceptible to the Bet:But extract. The presence of bactericidal concentrations (MBC) further supports its ability not only to hinder bacterial growth but also to eliminate these pathogens.

Notably, the strongest effect was observed against MRSA, which aligns with the results reported in the literature [5]. This can be explained by the structural differences between Gram-positive and Gram-negative bacteria [5]. Gram-negative bacteria, such as *P. aeruginosa*, possess a protective outer membrane consisting of lipopolysaccharides, phospholipids, and proteins, which has low permeability and contains hydrolytic enzymes that can hinder the effects of the phenolic compounds, followed by a thin layer of peptidoglycans [5]. In contrast, Gram-positive bacteria like MRSA lack an outer membrane and instead hold a thick, homogeneous peptidoglycan cell wall. Although this layer is thicker when compared to that of Gram-negative bacteria, it is more permeable, allowing phenolic compounds to penetrate more readily and exert antibacterial effects more effectively [5].

Kill Curves Assessment: Based on data from MIC and MBC, kill curve assays were conducted to evaluate the bacterial inactivation over time

Table 1
Concentrations of Bet:But extract ($mg_{GAE}.mL^{-1}_{solvent}$) and Bet:But 1:1 ES [% (v_{ES}/v_{total})] for MIC and MBC assays in each microplate well.

Well Number	1 (no bacteria)	2	3	4	5	6	7	8	9
[Bet:But extract] $mg_{GAE}.mL^{-1}_{solvent}$	2.79	1.39	0.696	0.348	0.174	0.0871	0.0435	0.0218	0.0109
[ES] % (v_{ES}/v_{total})	100	50.0	25.0	12.5	6.25	3.13	1.56	0.781	0.391

Table 2
MIC and MBC values for Bet:But extract ($mg_{GAE}.mL^{-1}_{solvent}$) and Bet:But ES [% ($v_{ES}/v_{total}$)] for both MRSA and *P. aeruginosa*.

Bacteria	Sample	MIC	MBC
MRSA	Bet:But Extract	0.174 $mg_{GAE}.mL^{-1}_{solvent}$	0.696 $mg_{GAE}.mL^{-1}_{solvent}$
	ES	50% (v_{ES}/v_{total})	Undetermined
P. aeruginosa	Bet:But Extract	0.696 $mg_{GAE}.mL^{-1}_{solvent}$	1.39 $mg_{GAE}.mL^{-1}_{solvent}$
	ES	25% (v_{ES}/v_{total})	Undetermined

for MRSA and *P. aeruginosa*, as shown in Fig. 3 (A and B). The aim was to understand how each bacterium would respond to extract and ES. To facilitate a more direct comparison of effects, the same concentrations were used for both bacteria. Therefore, the selected concentrations were 1.39 $mg_{GAE}.mL^{-1}_{solvent}$ for the Bet:But extract and the corresponding ES dilution [50% (v_{ES}/v_{total})], allowing for the assessment of their individual effects in the same proportion. This concentration was selected because it corresponds to the MBC for *P. aeruginosa*, representing the lowest concentration that could effectively inactivate both bacteria,

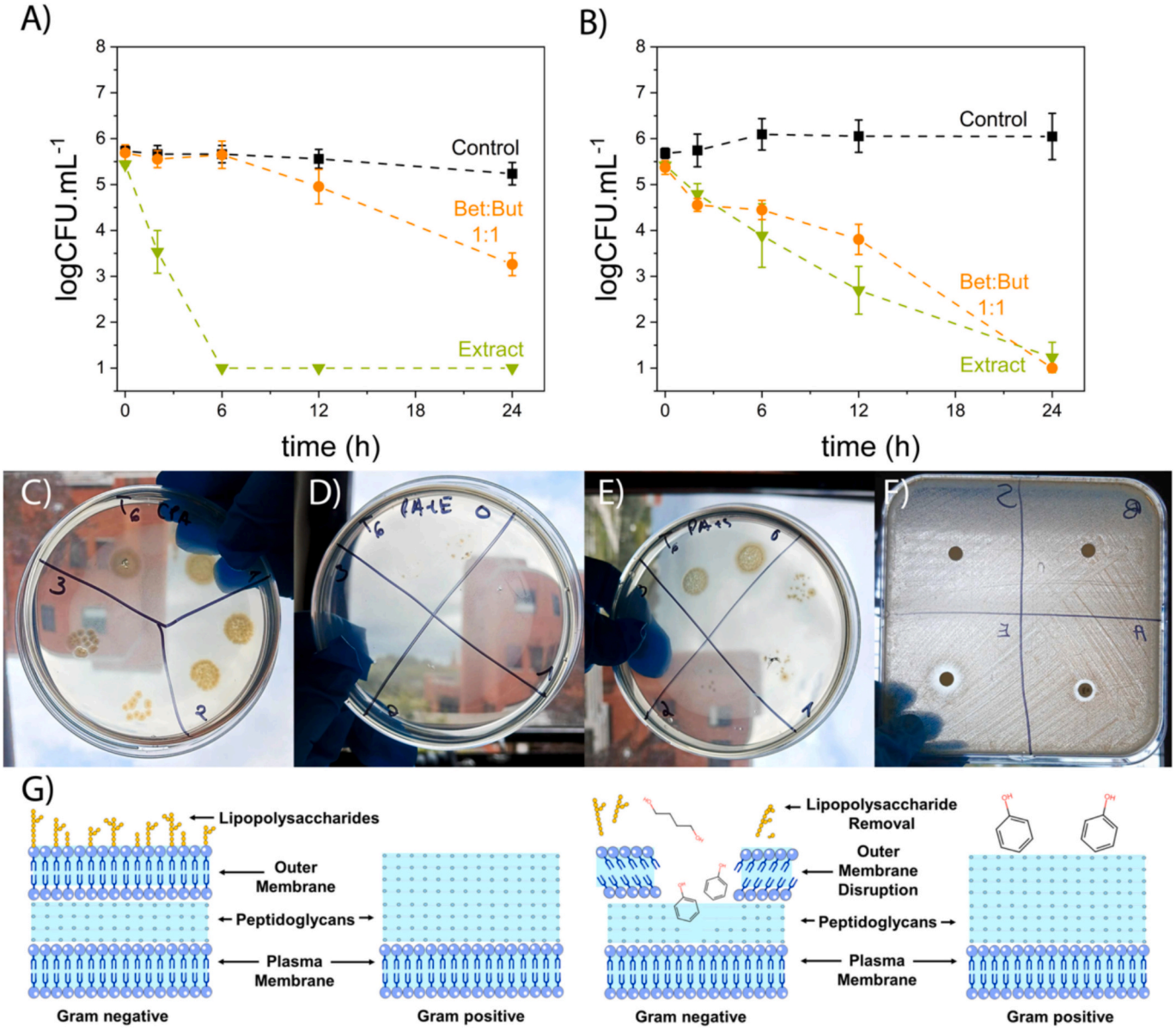

Fig. 3. *In vitro* biological assays. A) Inactivation of MRSA and B) *P. aeruginosa* after contact with Bet:But extract and ES, for 24 h, including a bacterial control. Values represent the mean of three independent assays and error bars represent the standard deviation. C) *P. aeruginosa* colonies at the 6-h point of the killing curve for bacterial control, D) bacteria with Bet:But extract, and E) bacteria with Bet:But 1:1 ES. F) Antibiogram results obtained for Bet:But 1:1 ES (top-left) and Bet:But extract (bottom left), including a blank disc as negative control (top-right), and a piperacillin disc (bottom-right) as positive control. G) Illustration of the cell membrane of Gram-negative *vs.* Gram-positive bacteria (left) and the proposed different action mechanism of the Bet:But 1:1 ES and Bet:But extract (right).

given that MRSA has a lower MBC value.

The results of the MRSA killing curve are depicted in Fig. 3A. It was observed that the Bet:But extract achieves inactivation of MRSA to the method's detection limit after 24 h, consistent with the previously obtained MBC of 0.696 $mg_{GAE}.mL_{solvent}^{-1}$. Notably, the Bet:But extract can inactivate MRSA to the detection limit within just 6 h of incubation (p-value <0.05), demonstrating a reduction of approximately 2 log CFU mL^{-1} after 2 h. Further corroborating the MIC and MBC assays, the ES shows a milder antibacterial effect, with a reduction of approximately 2.4 log CFU mL^{-1} from 0 to 24 h. These findings confirm that the antibacterial effect is primarily originated from the phenolic content present in the extract. At the same time, the bacterial control remained constant during the 24 h of the experiment (p-value >0.05).

The results of the *P. aeruginosa* killing curve are depicted in Fig. 3B. Both the extract and the ES achieved inactivation of *P. aeruginosa* to the method's detection limit after 24 h. This is consistent with MIC and MBC data, where MIC values are identical for both samples and the MBC value for the extract is 1.39 $mg_{GAE}.mL_{solvent}^{-1}$, suggesting a similar antimicrobial activity. However, unlike MRSA, the antibacterial effect against *P. aeruginosa* is primarily attributed to the ES. Although the extract shows slightly greater inactivation, these results suggest that the phenolic content in the extract has a limited impact on the inactivation of *P. aeruginosa*. While this may seem unusual, it is not without precedent, as ES has been reported to inactivate Gram-negative bacteria more effectively than Gram-positive strains. The underlying reasons for this remain unclear, and, since the effectiveness of ES depends on its HBA: HBD combination, the mechanisms may vary [5,11]. In this specific case, it is proposed that the observed effect is due to the use of 1,4-butanediol as the HBD. This compound has been previously reported to effectively remove lipopolysaccharides, thereby destabilizing the outer lipopolysaccharide membrane characteristic of Gram-negative bacteria and facilitating the passage of compounds through the relatively thin and permeable peptidoglycan layer, which contrasts with the thicker and less permeable layer found in Gram-positive bacteria [5,21,47]. Consequently, in Gram-positive bacteria, this solvent cannot disrupt their thick and homogeneous peptidoglycan layer, and thus, the phenolic compounds in the extract are primarily responsible for membrane disruption and bacterial inactivation [5]. However, other electrostatic interactions with *P. aeruginosa* cells may also contribute to these results [21,47]. Therefore, further studies are needed to fully understand the mechanisms driving this behavior.

Additionally, during this assay, a noticeable change in the colony shape of the bacteria was observed, particularly in *P. aeruginosa*, which produced much larger colonies than MRSA, making it easier to visualize the effect. In Fig. 3D and E, it is evident that the plates containing Bet:But extract (3D) and ES (3E) present smaller colonies compared to the bacterial control (3C). This suggests that the extract and solvent not only eliminate the bacteria but also affect their growth. A significant difference in the inhibition profiles of the extract (3D) and the solvent (3E) is observed, with the extract presenting a much higher reduction in bacterial concentration.

Antibiogram Evaluation: To evaluate the effect of the extract on a solid medium, an antibiogram was performed. Based on the previously obtained results, this assay was performed only for MRSA. The antibacterial activity of the Bet:But extract, at the undiluted concentration of 2.79 $mg_{GAE}.mL_{solvent}^{-1}$, and the Bet:But 1:1 ES (undiluted) were evaluated using the disc diffusion method. This concentration was chosen to maximize the phenolic content in the extract. A blank disc and a disc with the antibiotic piperacillin (30.0 μg) were included as negative and positive controls, respectively (Fig. 3F). The results demonstrate that the extract maintains its antibacterial activity against MRSA, since it presents an inhibition zone with 1.31 cm. This performance exceeds that of the tested antibiotic, piperacillin, which produced an inhibition zone of 0.98 cm. However, it is important to note that the extract was used at a much higher concentration than the antibiotic. In contrast, the ES disc showed no inhibition zone, since, as previously demonstrated for MRSA, the ES is inert toward this bacterium.

3.3. Production of aerosol and antibacterial evaluation

To prepare the aerosol prototype, several formulation parameters were decided. Based on the antibacterial assay results and the required aerosol performance, the extract concentration was maintained at its original value of 2.79 $mg_{GAE}.mL_{solvent}^{-1}$ to fully utilize its antimicrobial potential. Aluminum packaging equipped with a deodorant-type actuator was chosen to ensure efficient spray delivery, as the Bet:But extract is a water-based formulation (50 wt% water). To enable aerosolization while preserving formulation stability, a neutral propellant gas was required; nitrogen (N_2) was therefore selected. The final formulation consisted of 60% Bet:But extract and 40% N_2, ensuring both effective spray performance and preservation of extract integrity. This choice ensures that the extract remains undiluted and free from chemical alteration. After finalizing the aerosol prototype (Fig. 4A), and based on the results previously obtained, the following studies were conducted exclusively against MRSA. To ensure that the Bet:But extract from propolis rich in phenolic compounds retained its antibacterial properties after being added to the aerosol, an assay was performed by applying one, two, and three sprays (approximately 100 μL *per* spray) on MRSA-inoculated TSA plates. The results depicted in Fig. 4B, indicate that no bacterial growth occurred in the sprayed areas, demonstrating that the aerosol production did not compromise the extract's antibacterial properties. Furthermore, an increasing trend in inhibition was observed while increasing the number of sprays, validating the effectiveness of the aerosol delivery method.

Bacterial Inactivation on porcine skin (*ex vivo* assays): *Ex vivo* assays can replicate certain *in vivo* conditions within a controlled environment while offering greater biological complexity [48,49]. Therefore, to assess its suitability for skin disinfection, the Bet:But extract aerosol was evaluated *ex vivo* on porcine skin against MRSA. To evaluate the antibacterial potential of the aerosol, the assay was performed using the minimum effective amount, an equal volume of aerosol and bacterial suspension (approximately 200 μL). Additionally, an aerosol containing only the Bet:But 1:1 ES was used as a control. The results (Fig. 4C) showed a 2-log reduction in bacterial growth for the skin treated with the Bet:But extract based aerosol when compared to the untreated bacterial control after 24 h, as evidenced by statistically significant differences (p-value <0.05). The observed level of inhibition is lower than that seen in the *in vitro* assays, which is expected due to the greater biological complexity of *ex vivo* models, which typically lead to reduced antimicrobial efficacy [48,49]. Consistent with the *in vitro* assays, some bacterial inactivation was also observed with the aerosol based only in the ES, though the effect was significantly less pronounced than with the extract. The ES achieved less than a 1-log reduction and showed no statistically significant difference from the bacterial control (p-value >0.05). At 0 h, no significant differences between the samples were found (p-value >0.05), indicating that the extract does not immediately eliminate bacteria. However, the reduction observed over the 24-h period suggests that the extract becomes more effective once absorbed into the skin. These findings indicate that the aerosol based on the Bet:But extract, containing the phenolics recovered from propolis, is suitable for treating skin infections. Unlike conventional ethanol-based solutions, it does not evaporate as quickly, allowing for prolonged antibacterial action, which can find potential applications in wound dressings and infection prevention, particularly in inhibiting bacterial growth over time. Furthermore, as porcine skin models are well-established analogues for human skin [48,49], these results support the feasibility of using the phenolic Bet:But extract based aerosol for human skin infection treatments.

3.4. Cytotoxicity assays

Since the aerosol is intended for skin application, the cytotoxicity of

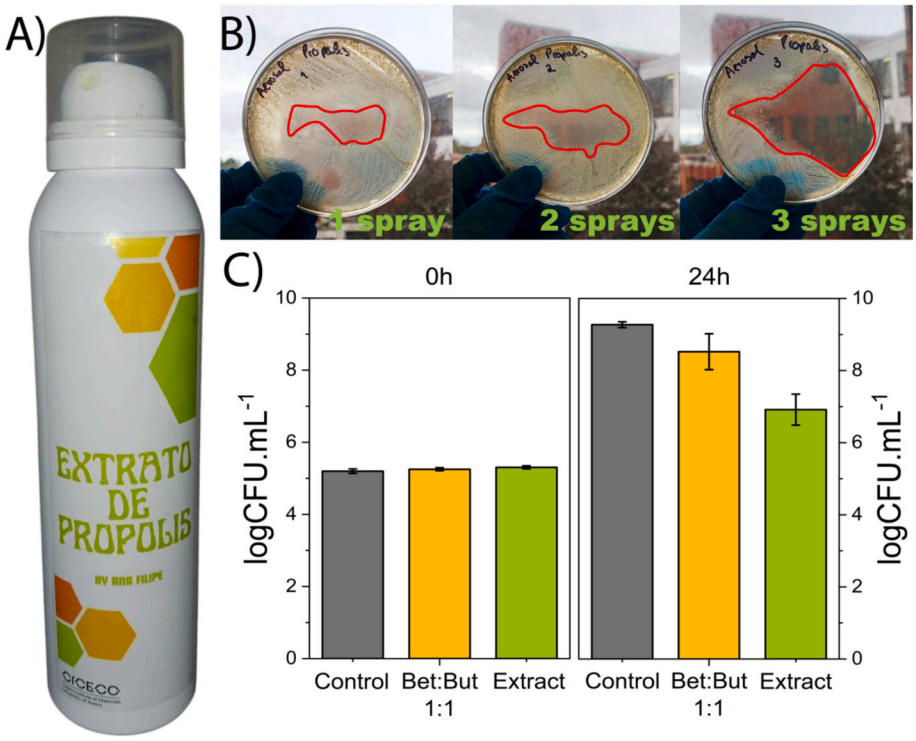

Fig. 4. Aerosol studies: A) Aerosol prototype. B) Growth of MRSA in plates sprayed with the aerosol, with 1, 2 and 3 aerosol sprays. C) *Ex vivo* inactivation of MRSA with the aerosols: Bet:But extract based aerosol and Bet:But 1:1 ES aerosol on porcine skin for 24 h. Values represent the mean of three independent assays, with two replicates each, and error bars represent the standard deviation.

the extract and the solvent was evaluated using human skin cells as an initial safety assessment. The spontaneously immortalized HaCaT cell line was chosen for this study, as it closely mimics normal human keratinocytes in terms of growth and differentiation characteristics, making it a suitable model for dermal toxicity screening [50]. The results shown in Fig. 5, demonstrate a concentration-dependent decrease in cell viability. The highest extract concentration tested (final concentration of 37.5 $\mu g.mL^{-1}$) resulted in a mild reduction in cell viability to approximately 70%. For all extract concentrations, similar cell viability values were observed for the corresponding amounts of the solvent (Bet:But 1:1 ES), with no statistically significant differences between the extract and solvent samples. This finding indicates that the observed reduction in cell viability is primarily attributed to the intrinsic mild cytotoxicity of the ES rather than to the phenolic compounds derived from propolis in the aerosol formulation.

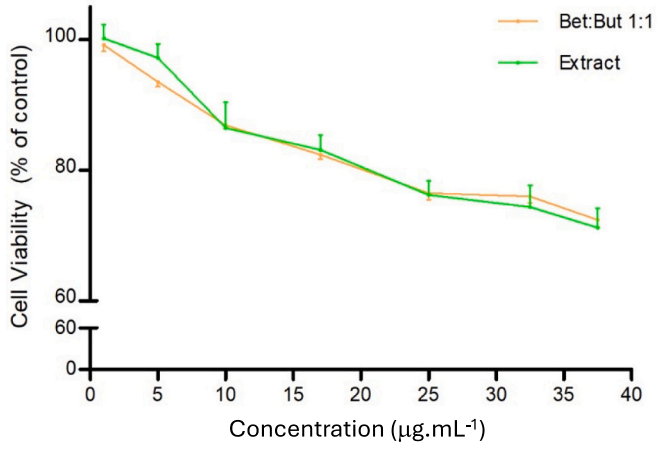

Fig. 5. Impact of the solvent (Bet:But 1:1 ES) and phenolics-based Bet:But extract on the viability of HaCaT cells after 24 h of exposure, evaluated using the MTT assay. Results are presented as mean ± SD, with the viability of untreated cell cultures considered as 100% ($n = 3$).

4. Conclusions

In this work, an aqueous-based antibacterial aerosol prototype, using an extract rich in phenolic compounds from green propolis recovered by an ES, was successfully developed. The propolis extract was obtained through extraction with Bet:But (1:1 M ratio), with 50 wt% of water content, yielding 139 ± 15 $mg_{GAE}.g^{-1}_{propolis}$. Rheological analysis indicated that the extract possessed an adequate viscosity to be applied in a typical aerosol formulation. The propolis extract effectively inactivated MRSA and *P. aeruginosa*, presenting MICs of 0.174 $mg_{GAE}.mL^{-1}_{solvent}$ (MRSA) and 0.696 $mg_{GAE}.mL^{-1}_{solvent}$ (*P. aeruginosa*), and MBCs of 0.696 $mg_{GAE}.mL^{-1}_{solvent}$ (MRSA) and 1.39 $mg_{GAE}.mL^{-1}_{solvent}$ (*P. aeruginosa*). At a concentration of 1.39 $mg_{GAE}.mL^{-1}_{solvent}$, it inactivated the bacteria to the detection limit of the method within 6 h for MRSA, and 24 h for *P. aeruginosa*. Finally, the undiluted propolis extract (2.79 $mg_{GAE}.mL^{-1}_{solvent}$) was incorporated into an aerosol formulation consisting of 40% of N_2 and 60% of the phenolic-based Bet:But extract. The aerosol retained its antibacterial properties, achieving a 2-log reduction in *ex vivo* assays on porcine skin against MRSA. Further studies with the aerosol on porcine skin are needed to improve the effectiveness of the inactivation. Cytotoxicity studies indicated that neither the solvent nor the extract present overt toxicity to human keratinocytes, suggesting the product's suitability for topical application. Although no direct skin–barrier interaction studies were conducted in this work, the ES is composed of betaine and 1,4-butanediol, both commonly used in topical and cosmetic formulations, and the final aerosol is highly diluted in water, suggesting a low risk of skin barrier disruption under the tested conditions. Likewise, comparisons with commercial extracts were not included in this study, however, such evaluations are planned for future work. These findings serve as a proof of concept, demonstrating both the antibacterial efficacy of the propolis aerosol, attributable to its phenolic

compounds, and its potential for treating skin infections.

CRediT authorship contribution statement

Ana R.F. Filipe: Writing – original draft, Methodology, Investigation. **Letícia S. Contieri:** Writing – original draft, Methodology, Investigation. **Márcia Braz:** Writing – review & editing, Methodology, Investigation. **Bárbara M.C. Vaz:** Writing – review & editing, Validation, Methodology. **Ana S. Fernandes:** Writing – review & editing, Funding acquisition, Conceptualization. **Ana Júlio:** Writing – original draft, Methodology, Investigation. **Maurício A. Rostagno:** Writing – review & editing. **Adelaide Almeida:** Writing – review & editing, Validation, Resources, Funding acquisition. **Leonardo M. de Souza Mesquita:** Writing – review & editing, Conceptualization. **Vitor Sencadas:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization. **Sónia P.M. Ventura:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

Data will be made available on request.

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