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Photodynamic inactivation of *Staphylococcus aureus* by ecological antibacterial solutions associating LED (ʎ 450 ± 10 nm) with curcumin and olive leaf extracts

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ABSTRACT

Antimicrobial resistance is a problem in contemporary society, with *Staphylococcus aureus* standing out as a threat due to its ability to colonize, its pathogenicity, and its expression of several virulence factors. In this context, antimicrobial photodynamic inactivation (aPDI) emerges as an alternative to conventional microbicidal or microbiostatic systems, enabling numerous and successive applications without developing side effects and microbial resistance. In this context, an aPDI system against cultures of *S. aureus* based on a water-in-oil (W/O) emulsion incorporating curcumin as the photosensitizer (PS), with and without olive leaf extract (OLE), was developed and the antibacterial efficacy evaluated under LED activation (ʎ450 ± 10 nm) by depositing an energy density of 14 J/cm^2. The produced emulsified systems showed no significant differences in the droplet size and morphology, remaining stable along the tested period of 30 days. The bacterial reduction achieved after the first aPDI application for the emulsions added with curcumin and curcumin combined with the OLE was 5 log10 CFU.mL^{-1} and 6 log10 CFU.mL^{-1}, respectively, revealing a significant difference between the two groups ($p < 0.0001$). After the second aPDI application, an increased microbial reduction (7 log10 CFU.mL^{-1}) was observed for both studied groups even with a low significant difference ($p < 0.05$). The PS loading through an emulsified system for aPDI obtained a bactericidal action against *S. aureus*, increased by applying two aPDI, showing a significant synergy between photodynamic inactivation, OLE delivery and antibacterial activity. In addition, the developed solutions were produced using natural products by an ecologically correct process.

1. Introduction

Antimicrobial resistance represents a major challenge and has been declared a significant problem worldwide. According to the Global Report on Surveillance of Antimicrobial Resistance, updated in 2015 by the World Health Organization, this is a growing threat to public health and a source of concern in various sectors of society [1,2]. Studies also report the association of antimicrobial resistance with an increase in morbidity, mortality, and costs, namely the risk of loss of efficacy and options in this class of drugs [3–5].

Among the kinds of bacteria under vigilance and study is the *Staphylococcus aureus*. Its colonization and pathogenicity capacity are a consequence of its virulence factors, and its genome presents resistance to traditional antibiotics, inferring various factors and means of evading the host's defences [6,7]. In addition to the ease multiplication and dissemination, *S. aureus* produces molecules with high pathogenic power that include enzymes and toxins such as beta-lactamases, coagulases, hyaluronidases, catalases, DNAses, lipases, proteases, esterases, among others [8,9]. The culture of *S. aureus* on exposed surfaces can survive for months, often forming biofilms, with greater antimicrobial resistance affecting public health and the agri-food industries [10,11].

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The antimicrobial photodynamic inactivation (aPDI) emerges as an alternative method to conventional microbicidal or microbiostatic systems, with the advantage of being applied numerous times without side effects and generation of microbial resistance [12]. It requires the use of a non-toxic photosensitizer (PS), capable of absorbing photons at a specific wavelength [13]. The activated PS can react with molecules in its vicinity by electron or hydrogen transfer, generating free radical production (type I reaction) or by transferring the energy excess to molecular oxygen (type II reaction), leading to the production of singlet oxygen ($^1O_2$) with high oxidizing power [14,15]. The response of the reactive oxygen species (ROS) with organic molecules is not specific; thus, any cellular component can be targeted by an aPDI. In fact, the diversity of targets that can be reached by the aPDI method makes it difficult to develop microbial resistance [16,17].

In this new era where sustainability is impacting our daily lives, including the industrial manufacturing, the use natural ingredients with less toxicity to substitute synthetic counterparts, together with the use of ecologically correct processes, is becoming a driving force guiding the search for alternative solutions. In this context, natural colorants like curcumin can play the role of PS in an aPDI system. Curcumin is a natural substance derived from the rhizome of turmeric (*Curcuma longa* L.) presenting in its structure several functional bioactive groups, which may result in potentiated effects when associated to light, with simultaneous reduced side effects [18–21]. To render curcumin more effective, compatibilization and delivery strategies have been studied to increase the water compatibility and bioavailability. These include encapsulation in liposomes, polymeric nanoparticles, emulsified systems, cyclodextrins, and solid lipid particles, which in addition to improve properties and efficiency can enable a safer and prolonged effect [22–25]. Among the possibilities, water-in-oil (W/O) emulsions have already proven their potential for a wide range of applications, such as cosmetic, pharmaceutical, agricultural, food, tanning, and paint industries. These systems can be used to carry hydrophilic active ingredients protected in the aqueous inner phase, but also to combine hydrophilic and hydrophobic compounds, exploring their synergic action, e.g., to potentiate the antimicrobial and antioxidant activity [26–31].

Face to the interest on eco-friendly antibacterial solutions with minimized side effects and microbial resistance, this study aims to develop a sustainable and green alternative for *S. aureus* inactivation based on an aPDI system activated by LED light. To achieve this goal, curcumin (a hydrophobic natural PS) was combined with a hydrophilic extract (olive leaf extract, OLE) using a biphasic system, namely a W/O, as the carrier system. By this strategy it is expected to achieve a synergetic action between curcumin and the OLE, with a consequent optimized efficacy of the antibacterial activity. Olive leaf is a residue from the olive oil productive chain, constituting an abundant plant material with high potential to develop value-added products within a sustainable circular bioeconomy [32]. In the olive oil industry, olive leaves represent approximately 10% of the olive fruit weight [33,34]. In olive cultivation, 25 kg of by-products (twigs and leaves) are produced per tree annually [35]. In this context, this work presents an innovative concept in which a natural dye (curcumin) is encapsulated in an emulsified system with OLE and used as PS in aPDI. Due to its hydrophobicity, and in order to be more accessible, it was envisaged a system where curcumin was positioned in the external phase of a W/O emulsion, added by a hydrophilic extract (OLE) incorporated in the internal aqueous phase. Apart from the novel developed aPDI system, this work also contributes for the valorisation of an agro-industrial residue. To the best of our knowledge this work was the first one addressing the use of W/O emulsions as base carriers for two applications of aPDI.

2. Material and Methods

2.1. Emulsions Preparation

Three emulsions have been prepared, namely a base emulsion (i.e., an emulsion without any addition; to be designated as "Emulsion"), an emulsion added with curcumin (to be designated as "Emulsion+curcumin"), and an emulsion combining curcumin (Sigma-Aldrich, Germany) and an olive leaf extract (OLE) (standardized extract with 20% of oleuropein, Essência d'um segredo, Arrentela, Portugal) (to be designated as "Emulsion+curcumin+OLE"). The emulsions were prepared using a W/O ratio of 40/60 (*v*/v). The general procedure to prepare the emulsions was according to a methodology described elsewhere [36]. Briefly, to prepare the sample "Emulsion", the aqueous phase (distilled water) was dispersed in the oil phase (corn oil, Fula, Algés, Portugal) containing Polyglycerol polyricinoleate (PGPR) (Palsgaardvej, Juelsminde, Denmark) at a concentration of 5 wt% (oil-basis), then homogenized using an Ultra-Turrax system (Unidrive X1000 Homogenizer Drive - CAT Scientific, Staufen, Germany) at 20000 rpm for 5 min. To prepare the sample emulsions "Emulsion+curcumin" and "Emulsion+curcumin+OLE" the oil phase was previously added with the curcumin powder at a concentration of 0.275% (*w/v*, oil-basis), followed by the addition of the PGPR (5 wt%). The mixture was stirred at 600 rpm using a magnetic stirrer (Rslab-1C, RSLab, Heraklion, Greece) Forlab, over four hours to achieve homogenization. For the sample "Emulsion+curcumin+OLE" the aqueous phase was previously prepared by adding the OLE at concentration of 2.5% (*w/v*, water-basis), thereafter mixed using the previously mentioned magnetic stirrer for 15 min at 600 rpm, then filtered through a Ø185 mm medium flow filter, 100/pk (Prat Dumas France) before use. The total concentration of curcumin in the emulsions was 3.19 mg/mL.

2.2. Emulsion Characterization and Stability

The prepared emulsions ("Emulsion", "Emulsion+curcumin", and "Emulsion+curcumin+OLE") were analysed concerning droplet size and morphology. Moreover, and having in view checking the impact of adding the curcumin and the OLE to the base emulsion, stability along time was also evaluated.

Emulsions droplet size and morphology was accessed by optical microscopy (OM) using an optical microscope (Nikon Eclipse Ni—U, Tokyo, Japan) equipped with a digital camera and NIS-Elements Documentation software. After images acquisition, the average droplet diameter was determined by computing 30 droplets from each sample. OM was also used to check morphological changes during the stability studies.

Stability along time was evaluated for a 30-days' time-frame period (inspection at 0, 10, 20, and 30 days) following the methodology described by Choudhary et al. [37] with minor adaptations. Briefly, the emulsions were transferred to a glass flask to fill a total height of 3 cm, thereafter stored at 4 °C and the creaming formation along time inspected. To complement this analysis for each time OM analysis was also done.

2.3. Bacterial Culture

The microbial culture of *Staphylococcus aureus* ATCC 6538 (Mistracon, Barcelona, Spain) stored in the ultrafreezer (ThermoFisher, STP, AS) at −70 °C was activated in Brain Heart Infusion (BHI) broth (Liofilchem, Roseto Degli Abruzzi, Italy) and incubated in a bacteriological oven (Raypa, Incutterm, Barcelona, Spain) at 37 °C for 24 h. Subsequently, 10% of the inoculum was added to the BHI broth and let to grow for 24 h at 37 °C in the exponential growth phase. In order to determine the mean inhibitory concentrations (IC_{50}) and carry out the Antibacterial Photodynamic Inactivation study, the microbial concentration was standardized to 1.8×10^{11} cells/mL by spectrophotometry (Jasco, V-730

UV–Visible, Tokyo, Japan) at a wavelength of 625 nm [38].

2.4. Emulsions IC_{50} Determination

From the original emulsions "Emulsion+curcumin", and "Emulsion+curcumin+OLE" (curcumin concentration of 3.19 mg/mL) dilutions with sterile distilled water were prepared to achieve samples with a curcumin concentration of 0, 40, 200, 600, and 1200 μg/mL to determine the IC_{50} in cultures of *S. aureus* standardized by optical density (1.8×10^{11} cells/mL) [17]. Microdilutions were carried out for the colorimetric assay with p-iodonitrotetrazolium chloride (INT) [39], then inoculated in Petri dishes with BHI agar culture medium (Liofilchem, Roseto Degli Abruzzi, Italy) to quantify the forming units of colonies per milliliter (CFU/mL) after 24 h of incubation at 37 °C [40].

2.5. Light Emission Procedure

Irradiations were done in continuous mode using a LED device (Emilight, MMOptics, São Carlos, SP, Brazil) with a power of 100 mW, at wavelengths of ʎ450 ± 10 nm, and by depositing an energy density of 14 J/cm^2 (Table 1). An irradiation angle of 90° and a distance of 1 cm were used. (See Fig. 1.)

The LED device was calibrated, and the energy absorption of the BHI broth medium was evaluated, through a potentiometer (Thorlabs Power Meter Sensor PM 30, Newton, New Jersey, United States) to establish the energy density to be delivered [41].

2.6. Antimicrobial Photodynamic Inactivation

Nine study groups were established to evaluate the developed aPDI system based on the W/O emulsions incorporated with curcumin at 31.9 μg/mL [42], with and without OLE. The emulsified systems were added to standardized *S. aureus* cultures in the exponential phase (1.8×10^{11} cells/mL), and the corresponding groups irradiated by LED. The experimental groups were as follows: (I) *S. aureus* culture (control); (II) *S. aureus* culture and "Emulsion" (E); (III) *S. aureus* culture and "Emulsion+curcumin" (E_{Cur}); (IV) *S. aureus* culture and "Emulsion+curcumin+OLE" ($E_{Cur\text{-}OLE}$); (V) *S. aureus* culture irradiated by LED (LED); (VI) *S. aureus* culture and "Emulsion+curcumin" irradiated by LED ($aPDI_1E_{Cur}$); (VII) second aPDI protocol application to VI 3 h after from the first one ($aPDI_2E_{Cur}$); (VIII) *S. aureus* culture and "Emulsion+curcumin+OLE" irradiated by LED ($aPDI_1E_{Cur\text{-}OLE}$); (IX) second aPDI protocol application to VIII 3 h after from the first one ($aPDI_2E_{Cur\text{-}OLE}$) (Table 2). All experiments were conducted in triplicate, and the pre-irradiation time used in the aPDI was 5 min. Then, the microdilution method and colorimetric assay with INT were applied [39]. From each dilution, 80 μL were inoculated in Petri dishes with BHI agar medium and incubated in a bacteriological oven (Raypa, Incutterm, Barcelona, Spain) at 37 °C for 24 h for subsequent quantification of the CFU.

2.7. Statistical Analysis

The results obtained in the different tests were analysed using ANOVA statistical test with Tukey's multiple comparison post-test using the GraphPad Prism® 8.0 software (San Diego-CA, USA).

Table 1
Light emission parameters used on the present study.

Parameter	LED
Wavelength (nm)	450 ± 10
Energy density (J/cm2)	14
Emission	CW
Spot size (cm^2)	9
Power density (mW)	100

3. Results and Discussion

3.1. Emulsion Characterization and Stability

Right after the production, all the emulsions presented similar morphology and average droplet diameter, 1.673, 1.743, and 1.645 μm, respectively for "Emulsion", "Emulsion+curcumin", and "Emulsion+curcumin+OLE", showing no significant differences among sizes, as expressed in Fig. 2. This indicates that no noticeable instability, namely coalescence phenomena, were observed due to the incorporation of the curcumin and the OLE. The droplets presented spherical shape and were homogeneously dispersed in the system. Studies by Rachmawati et al. [43] determined that curcumin in an emulsified system becomes more stable and protected from chemical degradation.

Along the 30-days period, the analysis by OM (Fig. 3) indicated signs of coalescence (increase in the droplet size due to the merging of the droplets), for the sample "Emulsion" (1.67 ± 0.29 to 4.86 ± 1.88 μm, respectively for t0 and t30) and the sample "Emulsion+curcumin" (1.74 ± 0.35 to 4.70 ± 1.16 μm, respectively for t0 and t30). This effect was negligible when the OLE was added (1.64 ± 0.26 to 1.88 ± 0.34 μm, respectively for t0 and t30), indicating the positive effect of the extract adding in the emulsion stability. In fact, according with published studies, the presence of flavonoids in the olive leaf extract, mainly luteolin 7-O-glucoside, rutin, apigenin 7-O-glucoside, luteolin 4'-O-glucoside and secoiridoids (oleuropein) [44], have been associated with the ability to reduce the O/W interfacial, decreasing the droplet size and enhancing the emulsion stability [45,46] corroborating the results achieved in this study.

At macroscopic level (Fig. 3) no creaming phase was detected, indicating that the level of coalescence was not enough to cause phase separation. Nevertheless, some curcumin sedimentation was observed for both the samples "Emulsion+curcumin" and the "Emulsion+curcumin+OLE". This could indicate an excess of curcumin in the external oil phase, even it is promptly re-dispersed in the system upon gentle stirring, pointing out the practical use of the developed system.

3.2. Emulsions IC_{50}

The cytotoxicity of the emulsions carrying curcumin, with and without OLE, "Emulsion+curcumin" and "Emulsion+curcumin+OLE", was analysed by determining the IC_{50} in cultures of *S. aureus* (Fig. 4).

It was found that the sample "Emulsion+curcumin" presented an IC_{50} of 68.19 μg/mL (Fig. 4A), a value higher than the one obtained for the sample "Emulsion+curcumin+OLE" (52.06 μg/mL) (Fig. 4B), which can be justified by the additional antimicrobial activity imparted by the OLE [47–49]. In fact, polyphenolic compounds, such as oleuropein, which is present in the OLE at a content of 20 wt%, are reported to present antimicrobial properties [50,51]. Moreover, studies carried out by Pereira et al. [50] identified that 7-*O*-methylnaringenin, a known flavonoid isolated from olive leaves, present antimicrobial activity against *S. aureus*. It should be noted that the use of extracts instead of the pure compounds is an attractive approach from an economic perspective. Moreover, their use can provide beneficial effects over the use of isolated compounds due to potential synergistic effects [52,53].

Several works have reported the determination of the minimum inhibitory concentration (MIC) of curcumin against *S. aureus*, but some discrepancies still remain. Mun et al. [54] showed that curcumin MICs against 10 strains of *S. aureus* (including 2 standard ATCC strains methicillin-resistant *S. aureus* (MRSA) and methicillin-susceptible *S. aureus* (MSSA), 4 clinical isolates of MRSA, and 4 MRSA from culture collection) ranged from 125 to 250 μg/mL while a study of Wang et al. [55] reported a MIC of 256 μg/mL against the MSSA. Additionally, Moghadamtousi et al. [56], who studied the antibacterial activity of an aqueous extract of *Curcuma longa* rhizome, obtained MICs ranging from 4 to 16 mg/mL for *S. aureus* cultures. Any of the described results indicate that concentrations below MIC will not limit the growth of

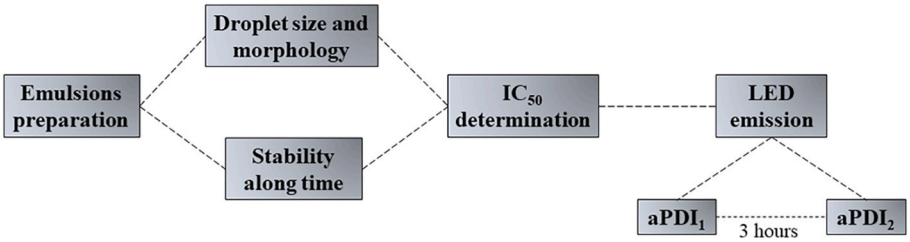

Fig. 1. Flowchart describing the methodology.

Table 2
Experimental groups evaluated in this study. (+) presente; (−) absent.

Experimental groups	*S. aureus*	Emulsion	Curcumin	OLE	LED
Control	+	−	−	−	−
E	+	+	−	−	−
E_{Cur}	+	+	+	−	−
$E_{Cur\text{-}OLE}$	+	+	+	+	−
LED	+	−	−	−	+
$aPDI_1E_{Cur}$	+	+	+	−	+
$aPDI_2E_{Cur}$	+	+	+	−	+
$aPDI_1E_{Cur\text{-}OLE}$	+	+	+	+	+
$aPDI_2E_{Cur\text{-}OLE}$	+	+	+	+	+

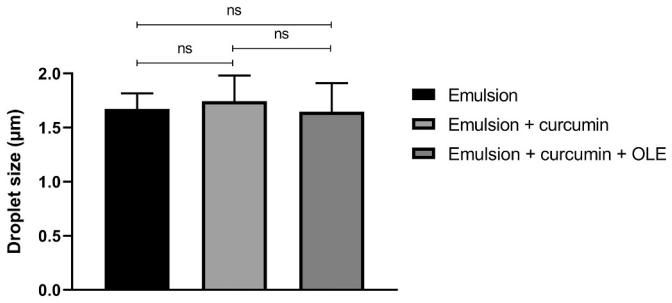

Fig. 2. Average droplet diameter. ns = not significant.

S. aureus, that is, antimicrobial effects will come from the generated stimuli such as PS activation by LED irradiation and consequent formation of ROS.

3.3. Antimicrobial Photodynamic Inactivation

For the antimicrobial photodynamic inactivation assays, the used curcumin concentration, 31.9 µg/mL, is below the IC_{50} value for both emulsified systems with curcumin ("Emulsion+curcumin", and "Emulsion+curcumin+OLE"). This fact corroborates that the obtained bacterial reduction values can be associated with the LED light emission and consequent activation of the PS with the consequent production of nonspecific ROS capable of affecting a diversity of microbial targets, thus hindering the ability to develop microbial resistance [16,17].

No significant antimicrobial activity was observed for the E_{Cur} group, in accordance with the studies performed by Péret-Almeida et al. [57] using ethanolic extracts of turmeric powder, commercial curcumin, and turmeric essential oil at concentrations of 5 mg/mL. Moreover, according to Tønnesen et al. [58], the antimicrobial activity can be only detected when curcumin is used at a concentrated ranging from 6.75×10^{-7} to 8.20×10^{-6} M when exposed to visible radiation.

The analysis of the antimicrobial activity upon the application of the first aPDI, relative to the control (*S. aureus* culture), indicated that the $aPDI_1E_{Cur}$ and $aPDI_1E_{Cur\text{-}OLE}$ study groups had a bacterial reduction of 99.999% and 99.9999%, respectively, with a significance of $p < 0.0001$ for both studied emulsified systems, proving the occurrence of bactericidal action. Comparing the aPDI action of the two emulsion groups ($aPDI_1E_{Cur}$ and $aPDI_1E_{Cur\text{-}OLE}$), there is an increase in activity when the OLE was used, characterized by a significance of $p < 0.0001$ and a reduction of 1 log $CFU.mL^{-1}$ (Fig. 5).

Freitas et al. [59] have already shown in their studies that curcumin-mediated LED photodynamic therapy (± ʎ450nm) reduced the viability of the reference strain of *S. aureus* by 4 log10. In comparison, curcumin without light reduced its survival in 2 log10. In the present study, where curcumin was loaded in a base emulsion carrier, a reduction in the bacterial viability of 5 log10 $CFU.mL^{-1}$ was achieved for the $aPDI_1E_{Cur}$ group, pointing out the efficacy of the developed systems. Moreover, when the bioactive OLE is co-added into the emulsified system, a higher reduction in the viability of the *S. aureus* culture, namely 6 log10 $CFU.mL^{-1}$, was achieved, by applying only one aPDI. This fact corroborates the importance of the OLE addition to achieve a strong prompt antimicrobial effect.

The analysis of microbial quantification after the second aPDI showed a reduction of 99.99999% ($p < 0.0001$) for both emulsion groups ($aPDI_2E_{Cur}$ and $aPDI_2E_{Cur\text{-}OLE}$), compared to the control. This result supports that when two aPDI are applied, an increased antibacterial action is achieved. Pellegrini et al. [60], had already demonstrated promising results with the dual application of photodynamic therapy in clinical trials with patients affected by circumscribed choroidal hemangiomas, considering this methodology as first-line.

Regarding the $aPDI_2E_{Cur}$ and $aPDI_2E_{Cur\text{-}OLE}$ groups comparison, there is a significance of $p < 0.05$, supporting the hypothesis that a synergic effect between the photodynamic inactivation provided by the curcumin, and the antimicrobial potential of the OLE loaded in the inner aqueous phase of the emulsion, have increased the antibacterial activity (Fig. 5). The results of the second aPDI application also support the existence of a sustained antimicrobial effect of the developed solutions.

Results described by Rocha et al. [42] reported a reduction of 5.2 log10 $CFU.mL^{-1}$ in cultures of MRSA through LED photodynamic therapy (ʎ430 nm), using systems based on microemulsions with curcumin (30 $\mu g.mL^{-1}$), corroborating the results obtained in the present work with the $aPDI_1E_{Cur}$ group. Nonetheless, through the application of two aPDI, it was possible, in this work, to achieve an increased reduction in the viability of *S. aureus* cultures of 7 log10 $CFU.mL^{-1}$. Introducing the curcumin into the oil phase prevents its degradation, namely in alkaline pH environments, and increases its bioavailability [61,62]. This enables the system to develop, in the first phase, an aPDI process and, later, due to the release of the hydrophilic compound, setting a cascade of synergetic bioprocesses leading to a sustained antimicrobial activity.

As reported by Fu et al. [63], the use of emulsions can induce changes in the permeability of the microorganism's plasma membrane by the presence of surfactants, improving the adsorption of the antimicrobial compound on the cell surface with a consequent increase of the antimicrobial activity. Concomitantly, the oil phase droplets can also induce ruptures in the extracellular matrix of microorganisms, making them more permeable to PS, thus increasing the efficiency of the aPDI, promoting the generation of ROS during the photoactivation of curcumin. The permeabilization of the extracellular matrix also allows a greater access and action of the delivered bioactive compound [42], similarly to what happens with the OLE in the present study.

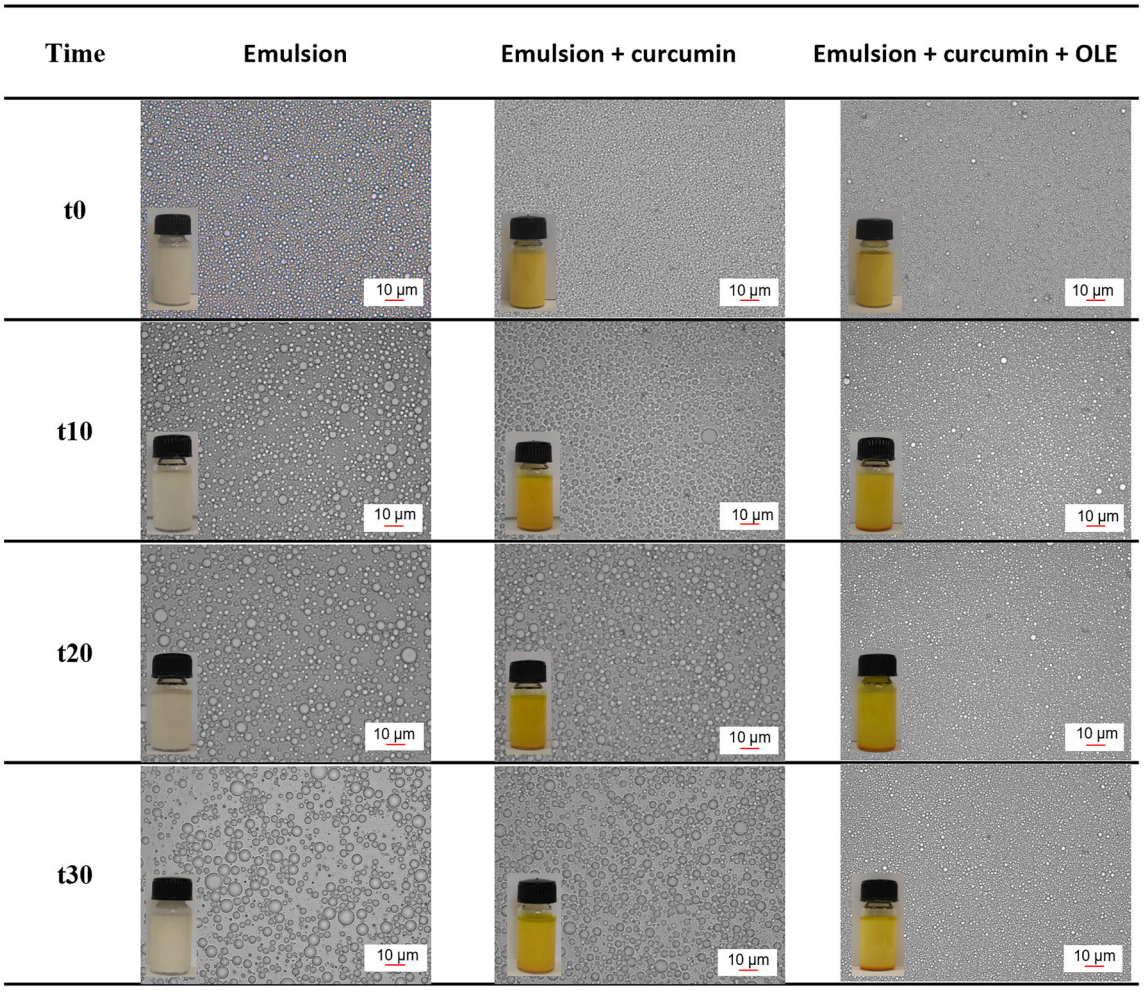

Fig. 3. Macroscopic and microscopic analysis (magnification of 400×) of the produced emulsions ("Emulsion", "Emulsion+curcumin", and "Emulsion+curcumin+OLE") along a period of 30-days.

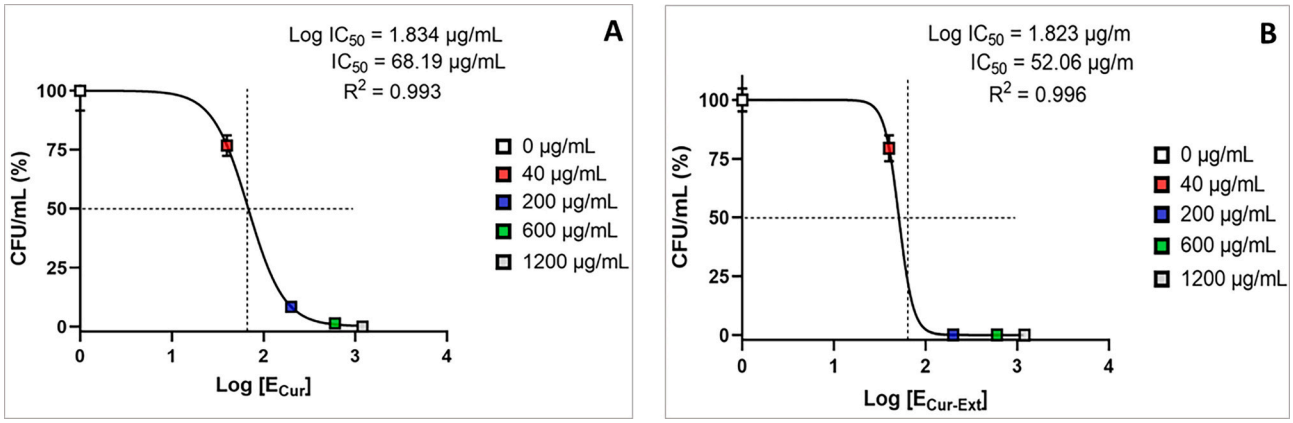

Fig. 4. Graphical representation of the mean cytotoxicity of the W/O emulsions in the presence of *S. aureus* cultures. (A) IC_{50} for "Emulsion+curcumin" (B) IC_{50} for "Emulsion+curcumin+OLE".

4. Conclusion

The approach of this study was based on the concept of synergistic photodynamic inactivation, with multi-directed action pathways to microbial substrates of *S. aureus*, characteristic of aPDI. The emulsified systems prepared (with and without OLE) showed no significant difference in size, remained stable for 30 days and maintained this stability under the thermal stress analysis imposed to 60 °C.

The first application of aPDI led to a loss of biological functionality of the *S. aureus* culture of 99.999% and 99.9999% in the $aPDI_{1ECur\text{-}Ext}$ and $aPDI_{1ECur}$ groups, respectively, proving a bactericidal action. There was a significance of $p < 0.0001$ between the emulsified groups, indicating an increase in the antimicrobial activity by the delivery of OLE. Through the second application of aPDI, a reduction in bacterial viability of

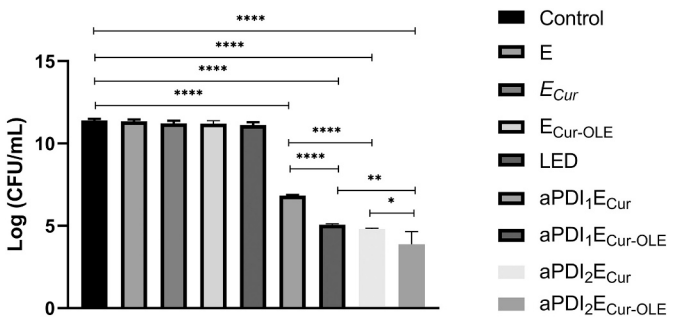

Fig. 5. Quantifications of *S. aureus* CFU in the different study groups. $^{****}p < 0.0001$; $^{***}p < 0.001$; $^{**}p < 0.01$; $^{*}p < 0.05$.

99.99999% was achieved along with confirmation of significance between the emulsified groups, proving the synergistic action between photodynamic inactivation and the delivery of the extract.

W/O emulsions have been shown to be effective as curcumin carrier agents for aPDI and OLE delivery, developing a sustainable antimicrobial process.

Author statement

We certify that this manuscript is original work and that it has not been published in any other medium and it is not under consideration for publication in any other journal. Furthermore, we the authors are liable for its content and for having contributed to the conception, design and implementation of the work, data analysis and data interpretation, and for having participated in writing and reviewing the text, as well as approving the final version submitted. In case of its acceptance, it will not be published elsewhere in the same form, in English or in any other language, including electronically without the written consent of the copyright-holder. The manuscript was checked for spelling and grammar. I also acknowledge that potential for conflict of interest does exist, as specified in the appropriate section in the manuscript.

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.

Data availability

No data was used for the research described in the article.

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