



Efficiency of the extracellular polymeric matrix disruptor Zerumbone in combination with Photodithazine® in the photodynamic inactivation of monospecies biofilms

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Abstract

Zerumbone (ZER), a sesquiterpene extracted from *Zingiber zerumbet* (L.) Smith, enhances the antibiofilm effects of antimicrobial photodynamic therapy (aPDT) by reducing the extracellular matrix of biofilms and increasing the generation of reactive oxygen species (ROS) when applied prior to aPDT. To reduce treatment application time, ZER was combined with Photodithazine® (PDZ) in a single solution. Then, this study investigated the potential of aPDT mediated by a mixture of ZER with PDZ in the inactivation of *Staphylococcus aureus*, *Escherichia coli*, fluconazole-susceptible (CaS) and resistant (CaR) *Candida albicans* biofilms. The solutions of ZER, PDZ and their mixture (ZER+PDZ) were analyzed using absorbance spectroscopy (AS), high-performance liquid chromatography (HPLC), and mass spectrometry (MS) to confirm the absence of molecular alterations in the mixture of ZER+PDZ. 48 h-biofilms were growth and treatments were performed: 1-ZER (256 µg/mL); 2-PDZ (200 µg/mL); 3-PDZ+LED; 4-ZER+PDZ+LED; 5- MIX (ZER+PDZ)+LED; Control (without treatment). Irradiation was performed using a red LED (660 nm, 50 J/cm², 30 mW/cm²). Sterile PBS was employed as the control group. For cytotoxicity assessments fffibroblast (FGF) and keratinocyte (NOK-si) oral cells were cultured for 24 h and submitted to treatments. Both ZER+PDZ+LED and MIX (ZER+PDZ)+LED groups showed the greatest statistically significant reduction in CFU/mL when compared to the control group ($p \leq 0.011$) in all evaluated strain, with no significant difference between them ($p \geq 0.218$). The reduction observed was 2.74, 2.89, 2.45 and 2.07 log₁₀ for *S. aureus*, *E. coli*, CaS, CaR, respectively. Cell viability reduction in NOK-si and FGF did not exceed 17%. AS, HPLC, and MS analyses demonstrated that PDZ retained its original characteristics following combination with ZER. Zerumbone combined with PDZ enhances the effect of antimicrobial photodynamic treatment regardless of strain characteristics and showed no cytotoxic effects.

Clinical trial number

Not applicable.

Keywords Photochemotherapy · Photosensitizing agents · Biofilms · Zerumbone

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Introduction

The oral microbiome is a complex ecological niche harboring up to 750 microbial species, including viruses, protozoa, archaea, fungi, and bacteria [1]. Microorganisms typically found in dentures are able to form polymicrobial communities, where various species interact in complex ways to influence biofilm composition and characteristics. The prevalence of *Candida albicans* and *Staphylococcus aureus* is higher in older adults compared to younger ones, making them common colonizers of denture bases [2].

C. albicans is associated with oropharyngeal candidiasis [3]. *S. aureus*, a Gram-positive coccus, can cause severe and virulent systemic infections, such as aspiration pneumonia, particularly in individuals with compromised immune function [4]. *S. aureus* and *C. albicans* causes bloodstream infections, representing a significant clinical challenge [4]. Additionally, in biofilms formed on implant-supported complete dentures, a positive correlation was observed between *Escherichia coli*, a Gram-negative coccus, and *C. albicans*, becoming the biofilm more complex [5, 6]. *C. albicans* frequently acts as a scaffold, facilitating the adhesion and growth of bacteria such as *E. coli* and *S. aureus* [7]. These interactions can be synergistic, enhancing both antimicrobial resistance and biofilm virulence [8].

Oral infections are frequently associated with the formation of biofilms, characterized for adherent cells on biotics and abiotic surface and protected by a self-secreted extracellular polymeric matrix (ECM) [2, 5, 8]. The ECM contributes to the structural integrity of the biofilm and impedes drug penetration, which is influenced by factors such as the constituent microorganisms, nutrient availability, substratum characteristics, and flow and oxygenation conditions [5, 6]. The inherent structure of biofilms has increased the emergence of resistant species [5, 8]. In addition, the prolonged or inappropriate use of antimicrobial drugs has been associated with antimicrobial resistance, and also cause adverse effects in organs like the liver and kidneys [3, 8]. These limitations underscore the need to develop novel antibiofilm therapies with optimized mechanisms of action, capable of overcoming microbial resistance and reducing toxicity.

Antimicrobial Photodynamic Therapy (aPDT) is an alternative approach for treating infectious diseases associated with biofilms [9]. The interaction of a photosensitizer with molecular oxygen is light-dependent; upon absorption of specific wavelengths [10, 11]. aPDT produces reactive oxygen species (ROS), including singlet oxygen, which are highly cytotoxic [10, 11]. These ROS can cause damage to various cellular components such as proteins, lipids, and DNA, ultimately leading to cell death [10]. Photodithazine® (PDZ), a chlorin e6 derivative with an absorption

peak at 660 nm, was selected as a photosensitizer due to its proven ability to generate reactive oxygen species upon light activation, resulting in effective antifungal activity. PDZ-mediated aPDT has been employed for the treatment of biofilms in in vitro, in vivo and clinical studies [12–15].

The biofilms' ECM represents a significant limitation for aPDT, since its structure can hinder the penetration of photosensitizers (PS) in deeper layers of biofilms [16]. Furthermore, the properties of PSs also play a crucial role in influencing the effectiveness of aPDT in biofilms [17]. Another limitation of photodynamic treatments is related to differences in the cell wall composition among different microorganisms, as Gram-positive bacteria are more susceptible to aPDT than Gram-negative bacteria [18]. The anti-biofilm process of aPDT occurs in two steps. First, the photosensitizer binds to the biofilm matrix and can be sequestered by the polysaccharides or partially penetrate to reach the microbial cells. Some photosensitizers adhere to the cell surface, while others penetrate the cytoplasmic membrane and reach organelles, such as the nucleus, in fungal cells. In the second step, upon illumination, ROS are generated, causing oxidative damage to several targets, including polysaccharides, cell surface lipids, intracellular proteins and DNA. This process leads to biofilm disintegration and cell death [10, 11]. Therefore, studies have evaluated ECM disruptor agents previously to aPDT, such as DNase I [13, 14], Zerumbone (ZER) [19–21], and oxygen peroxide (H_2O_2) [22] for the treatment of biofilms.

ZER, extracted from the rhizomes of *Zingiber zerumbet* (L.) Sm., is a sesquiterpene phytochemical that has attracted considerable scientific attention owing to its diverse pharmacological properties, notably its potent anti-tumor, anti-inflammatory, antioxidant, and antimicrobial activities [23]. ZER exhibits a multifaceted mode of action, interacting with various cellular pathways and molecular targets [24]. By promoting the production of ROS, ZER can trigger oxidative stress, ultimately leading to apoptosis in microbial cells. ZER has been employed as an ECM disruptor agent able to cause considerable reductions of biofilms' components, such as polysaccharides, extracellular DNA, proteins, biomass [19–21]. Pre-treatment with ZER at 256 $\mu\text{g/mL}$ prior to aPDT enhanced the antimicrobial effect of aPDT against fluconazole-susceptible and resistant *C. albicans* biofilms, resulting in a 2.43 $\log_{10}$ and a 2.04 $\log_{10}$ reduction, respectively [21]. These results are promising, once a single application of ZER followed by aPDT was able to induce a significant reduction in biofilms' components, suggesting a promising alternative treatment for drug-resistant biofilms.

An important limitation of the therapies that use disruptor ECM agents previously to aPDT is the prolonged time of application since demands two steps of application (ECM disruptor followed by photosensitizer) [13, 14, 20–25].

Therefore, the aim of this study was to evaluate if ZER mixed with Photodithazine® have the same positive effects when it's applied alone, optimizing the treatment time. The mixture of ZER and PDZ-mediated aPDT were evaluated in *S. aureus*, *E. coli*, fluconazole susceptible and -resistant *C. albicans* biofilms. In addition, the mixture was analyzed using absorbance spectroscopy (AS), high-performance liquid chromatography (HPLC), and mass spectrometry (MS) to confirm the absence of molecular modifications in the photosensitizer. The evaluation of cytotoxicity was also performed.

Metodology

Preparation of Zerumbone and Photodithazine® solutions

Zerumbone (ZER, $C_{15}H_{22}O$; Sigma-Aldrich, St. Louis, MO, USA) (Fig. 1A) was purchased as crystals and initially diluted in 1 mL of dimethyl sulfoxide (DMSO - Sigma-Aldrich, St. Louis, MO, USA). Serial dilutions were conducted from the initial solution using Mili-Q water (Milli-Q® IX 7003 - Sigma-Aldrich, St. Louis, MO, USA) until reach a final concentration of 256 $\mu\text{g/mL}$, previously determined [19]. For this, 25.6 μL of the initial solution was diluted in 974.4 μL of Mili-Q water [19]. Photodithazine® (PDZ, $C_{34}H_{36}N_4O_6 \cdot 2C_7H_{15}NO_5$; VETA-GRAND Co., Russia) (Fig. 1B) was applied at a concentration of 200 $\mu\text{g/mL}$ [12, 25], either individually or combined with ZER. PDZ was prepared in Phosphate-Buffered Saline (PBS; pH 7.2, Sigma-Aldrich, St. Louis, MO, USA) when applied individually, or in a ZER solution at 256 $\mu\text{g/mL}$ when used in combination. The chemical structure of ZER and PDZ were described in Fig. 1. This same preparation protocol was applied in all experiments. The solutions of ZER, PDZ and their combination (ZER+PDZ) were analyzed using absorbance spectroscopy (AS), high-performance liquid chromatography (HPLC), and mass spectrometry (MS) to confirm that no molecular alterations occurred in the substances upon mixing.

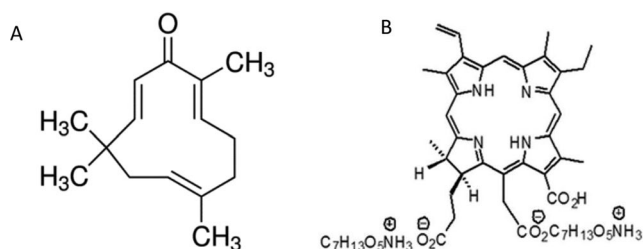


Fig. 1 Molecular structure of Zerumbone (A) and Photodithazine® (B) [12–15, 23]

Light sources

The light source used in this study was a red light-emitting diode (IrradLED®– Biopdi, São Carlos, SP, Brazil). The system of red LED (660 nm) has a homogeneous illumination area composed of 24 LED and a cooling device. The distance between the LED and the plate (3 cm) allowed an even distribution of light on each well. The illumination angle was maintained at a perpendicular orientation (90°) relative to the surface on which the plates were positioned [12, 25]. The light energy dose (fluence) was 50 J/cm^2 , delivered at a power density of 30 mW/cm^2 for 20 min (Fig. 2).

Microorganisms and culture conditions

In this study, reference strains from the American Type Culture Collection (ATCC, Rockville, USA) were used: *S. aureus* (ATCC 25923), *E. coli* (ATCC 25922), fluconazole-susceptible *C. albicans* (CaS; ATCC 90028) and fluconazole-resistant *C. albicans* (CaR; ATCC 96901). The strains were stored at -80°C and were previously reactivated for biofilm formation.

The reactivation of *E. coli* and *S. aureus* was performed using Tryptic Soy Agar (TSA; Sigma-Aldrich, St. Louis, MO, USA). The reactivation of *C. albicans* strains was performed using Sabouraud Dextrose Agar (SDA; Sigma-Aldrich, St. Louis, MO, USA) supplemented with chloramphenicol (0.05 g/L; Sigma-Aldrich, St. Louis, MO, USA). After 48 h of microorganisms' growth, five colonies of each strain were collected and suspended in tube containing 5 mL of Yeast Nitrogen Base medium (YNB; Sigma-Aldrich, St. Louis, MO, USA) for *C. albicans* strains; and Tryptic Soy Broth (TSB) for *E. coli* and *S. aureus* strains. The cultures were incubated at 37°C for 16 h (pre-inoculum). Following the 16-hour incubation, a 1:20 dilution (inoculum) was performed, and the tubes were incubated for 6 h for *S. aureus*, 1.5 h for *E. coli*, and 8 h for *C. albicans*, until reach the respective mid-log phase, previously determined based on growth curves [23, 26].

Biofilm formation

The inoculum was resuspended in its respective initial medium (YNB or TSB) with a volume of 8 mL. Spectrophotometric (Kasvi, Curitiba, PR, Brazil) readings were performed (OD 640 nm for CaS and CaR; OD 600 nm for *S. aureus* and *E. coli*) based on previous growth curves to achieve concentrations corresponding to 10^7 CFU/mL. The OD values corresponding to this concentration were: CaS (0.550 ± 0.04); CaR (0.480 ± 0.05); *S. aureus* (0.659 ± 0.04); *E. coli* (0.306 ± 0.01). For inocula that did not reach the OD corresponding to 10^7 CFU/mL after the first resuspension,

Fig. 2 Schematic representation of the LED equipment (IrradLED®– Biopdi, São Carlos, SP, Brazil). **(a)** Image of the LED equipment with a 24-well plate inside. **(b)** The LED apparatus illuminates a well within the plate, clearly showing the distance between the LED and the plate, the lens, the platform, and the specific well-being illuminated in the 24-well plate

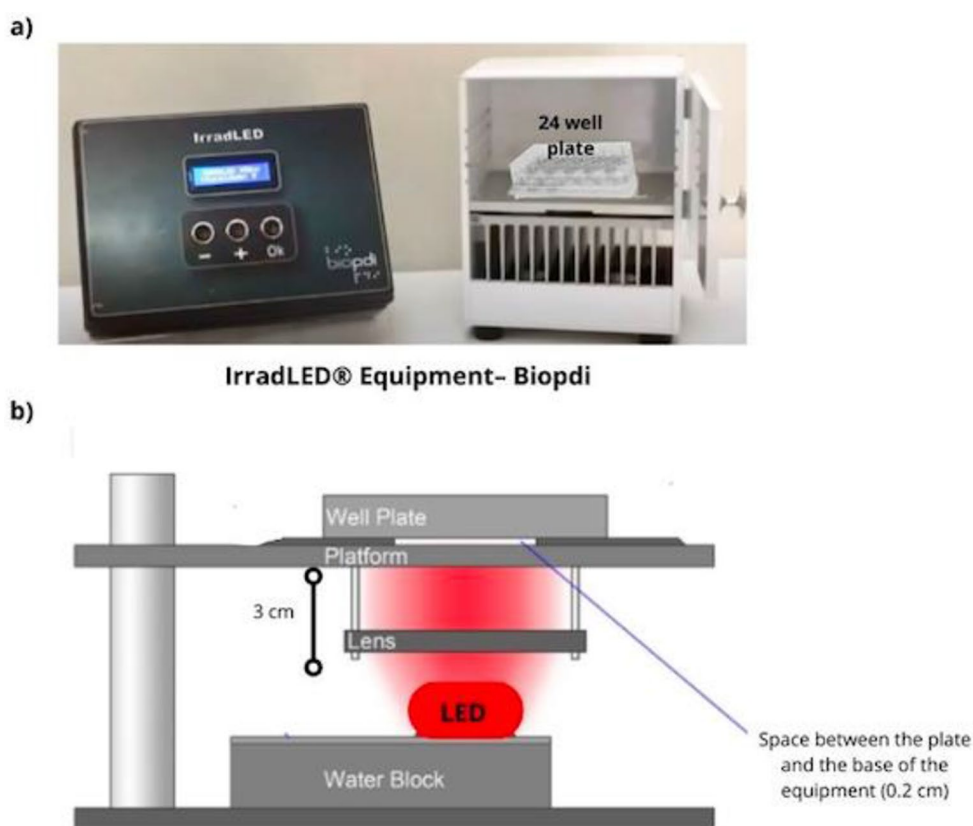


Table 1 Treatment parameters of 48 h mature biofilms

Groups	Description
Control	Biofilms were washed twice with sterilized PBS.
ZER	Biofilms were treated only with 500 μ L of ZER (256 μ g/mL) for 20 min [19].
PDZ	Biofilms were treated with 500 μ L of PDZ (200 μ g/mL) for 20 min [10,25].
PDZ+LED	Biofilms were treated with 500 μ L of PDZ (200 μ g/mL) for 20 min followed by LED light irradiation (50 J/cm ²) [10,25].
ZER+PDZ+LED	Biofilms were treated with 500 μ L of ZER (256 μ g/mL) for 20 min. After removal of the solution, 500 μ L of PDZ (200 μ g/mL) was applied for 20 min, followed by LED light irradiation (50 J/cm ²). was conducted (Abreu-Pereira et al., 2025).
MIX (ZER+PDZ)+LED	Biofilms were treated with the mixture of ZER (256 μ g/mL)+PDZ (200 μ g/mL) for 20 min, followed by LED light irradiation (50 J/cm ²).

an additional 500 μ L of the medium was added, and a new reading was performed until the working concentration was reached. Subsequently, 1 mL of each resuspension was individually transferred to 24-well cell culture plates (Kasvi, Curitiba, PR, Brazil) and incubated at 37 °C under agitation (75 rpm) for 90 min (adhesion phase).

Biofilms treatments

The 48 h-biofilms were treated as described in Table 1. Three independent experiments, performed in quadruplicate, were conducted for each experimental condition ($n=12$).

After the treatment, the samples were submitted to serial dilutions and 100- μ L aliquots that were spread over the

surface of TSA (*S. aureus* and *E. coli*) and SDA (*C. albicans*) plates. The plates were incubated aerobically at 37 °C for 48 h and colony-forming unit (CFU) were determined [25].

Cytotoxicity assay

Human Gingival Fibroblast (H GF– Cell Bank of Rio de Janeiro; code: 0089) and oral keratinocytes (NOK-si– kindly provided by Professor Carlos Rossa Jr., Department of Periodontology, Araraquara Dental School, UNESP) were maintained in 75 cm² flasks and grown until reach 70% of confluence. Cells were then washed twice with PBS, detached from the flasks using 0.05% (v/v) trypsin/0.53 mM

EDTA (Sigma-Aldrich, MO, USA), and centrifuged (2000 RPM, 5 min, 26 °C). Following centrifugation, cell pellets were resuspended in DMEM (high glucose—4.5 g/L; Sigma-Aldrich, St. Louis, MO, USA). Cell suspensions were adjusted to a concentration of 10^4 cells/mL by dilution with DMEM and reading on digital cell counter (Countess 3; Thermo Fisher Scientific, Waltham, Massachusetts, EUA), and 200 μ L aliquots were transferred to the wells of 96-well plates (Kasvi, Curitiba, PR, Brazil). The plates were incubated for 24 h at 37 °C under a 5% CO₂ atmosphere [27].

Following incubation, DMEM was removed, and treatments were conducted as follows in Table 1. Subsequently, 200 μ L of DMEM, and 20 μ L of alamarBlue™ solution (Thermo Fisher Scientific, MA, USA) were added to each well. After a further 16-hour incubation period, fluorescence intensity was measured using a fluorescence reader (FLUOstar Omega, BMG Labtech, Cary, NC, USA; Ex: 544–10 nm; Em: 590–10 nm) [27]. Experiments were performed in triplicate in three independent trials ($n=12$).

Absorbance spectrum

UV-VIS spectra of ZER solutions, PDZ or their combination (ZER+PDZ), were evaluated. The solutions were prepared using the same parameters previously described: ZER (256 μ g/mL), PDZ (200 μ g/mL) and a mixture in which PDZ was prepared in ZER solution (256 μ g/mL). Maximum absorbance peaks were recorded across the spectral range of 300 to 800 nm at each time point. Absorbance measurements for each peak were obtained using a Synergy H1 Multi-Mode Reader (BioTek, Winooski, VT, USA). Photobleaching kinetics were subsequently evaluated through graphical representation of the data.

High-performance liquid chromatography (HPLC)

The solutions (ZER, PDZ and ZER+PDZ) were prepared conforming described previously and stored in the absence of light to prevent photodegradation of the photosensitizer. Before purification, the samples were filtered through a 0.45 μ m filter. HPLC (high performance liquid chromatography) analysis were performed in an analytic mode in an equipment from Waters (Alliance/blue, Waters, Franklin, MA, USA) with a XBridge C18 column (size: 4.6 $\times$ 25 cm; particle size: 5 μ m) and with detection at 254 nm. Solvents employed for the analyses were trifluoroacetic acid (TFA) at 0.045% in water and 0.036% of TFA in acetonitrile (diluent solution; Sigma-Aldrich, St. Louis, MO, USA). The gradient used was from 5 to 95% solution (mobile phase) in 30 min, with a flow rate of 1 mL min⁻¹. Then, the characterizations

of the ZER and PDZ solutions (prepared in PBS or in ZER) were performed ($n=12$).

Mass spectrometry

Prior to each analysis, ZER, PDZ and ZER+PDZ solutions were prepared conforming described previously and stored in the absence of light. The molecular weights of the compounds were analyzed using a mass spectrometer in ESI-IT-MS configuration (LCQ Fleet; Thermo Fisher Scientific, Waltham, MA, USA), with an ion trap analyzer in positive electrospray mode (M^+H^+) and a range of 200–2000 g mol⁻¹. The mass spectrometry equipment was washed using a 1:1 solution of Milli-Q water and acetonitrile with 0.1% formic acid (Sigma-Aldrich, St. Louis, MO, USA). Spectra were obtained directly by injecting a solution (1:1) into the spectrometer using a 500 μ L syringe, allowing for the acquisition of a qualitative data spectrum through electrospray ionization (ESI), which generates gas-phase ions from each solution (ZER, PDZ or ZER+PDZ) for Mass Spectrometry analysis ($n=12$).

Statistical analysis

Data normality (Shapiro-Wilk test) and homoscedasticity (Levene's test) were assessed prior to further analysis. Data meeting these assumptions were analyzed using one-way ANOVA, with treatment type as the independent variable (group). Tukey's post-hoc test was used for homoscedastic data, while the Games-Howell test was employed for heteroscedastic data. The sample size for each group was $n=12$ for each dependent variable. A significance level of 5% was adopted. Statistical analyses were performed, in duplicate, using IBM SPSS Statistics for Windows, version 21 (IBM Corp.).

Results

Zerumbone improves the efficacy of antimicrobial photodynamic therapy

A statistically significant difference in CFU counts ($p<0.001$) was observed among treatment groups for all the evaluated strains: *S. aureus* (Fig. 3A), *E. coli* (Fig. 3B), CaS (Fig. 3C) and CaR (Fig. 3D).

For all strains, both the ZER+PDZ+LED and MIX(ZER+PDZ)+LED groups exhibited the greatest statistically significant reduction in CFU/mL counts ($p\leq 0.011$) compared to the control group with no significant difference between them ($p\geq 0.218$). The average reduction was

Fig. 3 Colony-forming units (CFU/mL) following treatments in biofilms of *S. aureus* (A), *E. coli* (B), CaS (C), and CaR (D). Different letters indicate statistically significant differences ($p < 0.05$) by Tukey's or Games-Howell post-hoc test ($\alpha = 5\%$)

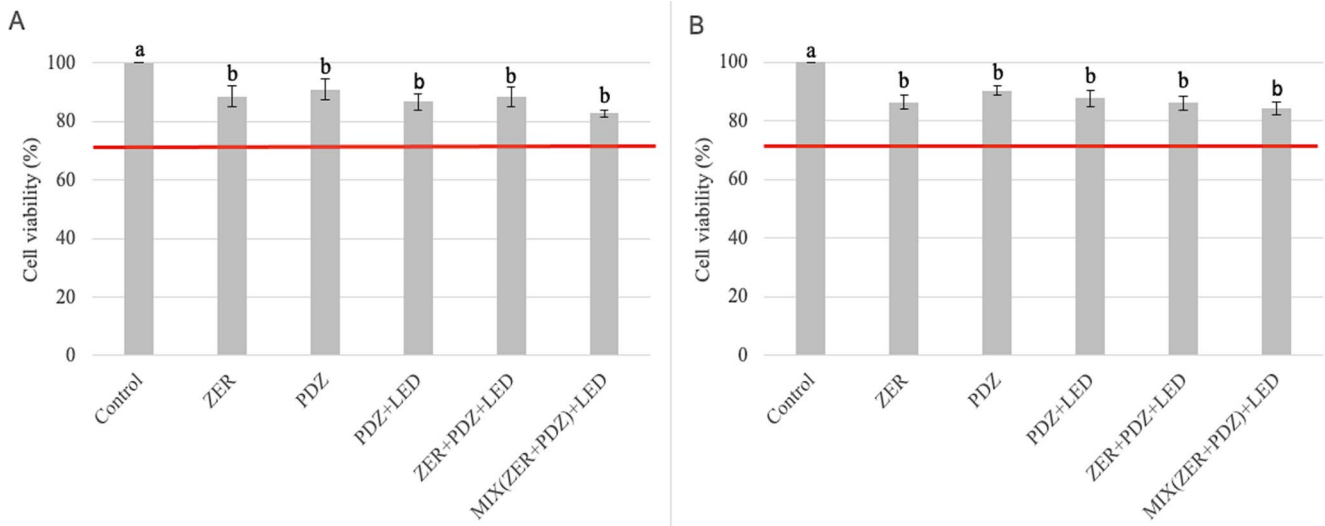
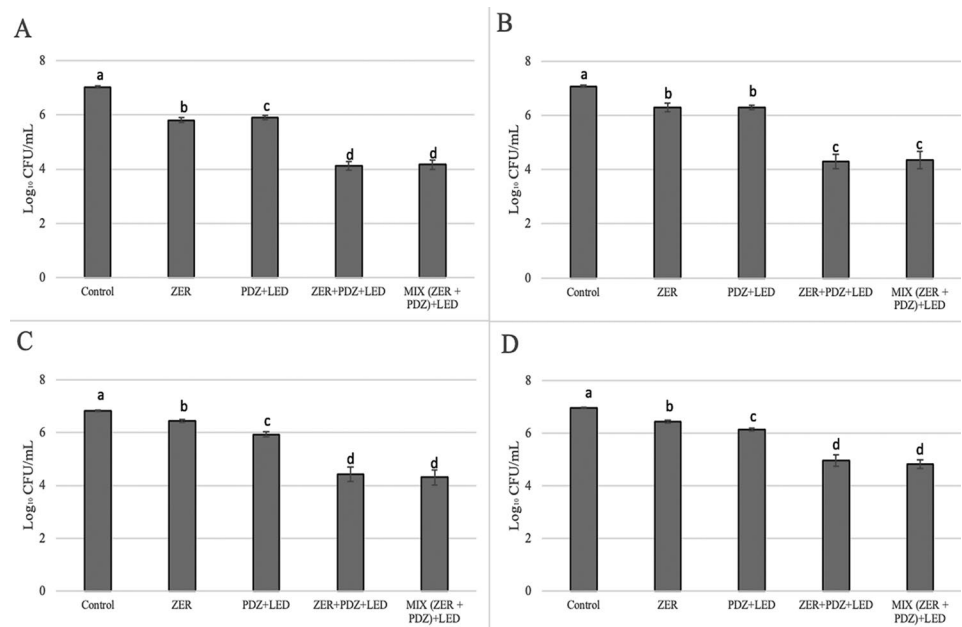


Fig. 4 Cytotoxicity of NOK-si (A) and HGF (B) cells following treatments. Different letters indicate statistically significant differences ($p < 0.05$) by Tukey's (A) or Games-Howell (B) post-hoc test ($\alpha = 5\%$). Red line indicates the acceptable limit for cell death (up to 30%)

2.74, 2.89, 2.45, 2.07 $\log_{10}$ for *S. aureus*, *E. coli*, CaS, CaR, respectively.

For *S. aureus* (Fig. 3A), the ZER and PDZ+LED groups showed the second highest significant reduction in cell viability ($0.77 \log_{10}$; $p \leq 0.011$) and were statistically similar to each other ($p = 0.076$). Across all strains, the PDZ group demonstrated a performance comparable to the control group ($p \geq 0.898$). For *E. coli* (Fig. 3B), the ZER group exhibited the second greatest significant reduction ($1.23 \log_{10}$; $p = 0.018$), followed by the PDZ+LED group ($1.13 \log_{10}$; $p = 0.030$), and these groups were statistically different ($p = 0.048$) from each other. For CaS (Fig. 3C) and CaR (Fig. 3D), the PDZ+LED group showed the second highest

significant reduction ($p \leq 0.001$) in cell viability ($0.88 \log_{10}$ for CaS and $0.81 \log_{10}$ for CaR; $p \leq 0.001$), followed by the ZER group ($0.37 \log_{10}$ for CaS and $0.52 \log_{10}$ for CaR; $p \leq 0.001$). These two groups were statistically different from each other ($p \leq 0.028$).

Cytotoxicity of treatments

A statistically significant difference ($p = 0.036$) was observed among treatment groups for NOK-si cells (Fig. 4A). All treatment groups were statistically different ($p \leq 0.046$) from the control group but were not significantly different from each other ($p \geq 0.075$). For HGF cells (Fig. 4B), a statistically

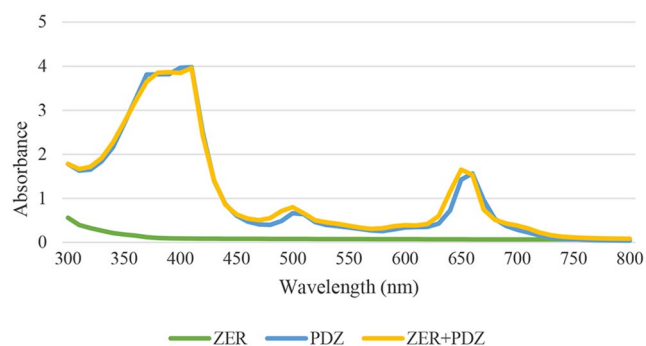


Fig. 5 Absorbance spectrum of the solutions

significant difference ($p=0.007$) was also observed among treatment groups. All treatment groups were statistically different ($p \leq 0.031$) from the control group were not significantly different from each other ($p \geq 0.127$). The reduction in NOK-si and FGH cells did not exceed 17%, which is within the acceptable limit of 30% (ISO, 2009).

Absorbance spectrum

The absorbance spectrum of the ZER and PDZ mixture exhibited the same characteristics as PDZ when analyzed individually (Fig. 5). ZER solutions exhibited undetectable absorbance across the measured spectrum, with no discernible peaks (Fig. 5). Statistical analysis revealed no significant difference in absorbance between the PDZ and PDZ + ZER ($p=0.905$). ZER was statistically different ($p=0.001$) from the other groups.

High-performance liquid chromatography (HPLC)

HPLC analysis of ZER (Fig. 6A), PDZ (Fig. 6B), and the ZER+PDZ mixture (Fig. 6C) revealed that the retention time characteristics of each analyte remained unchanged when their chromatographic profiles were overlaid (Fig. 6D). These consistent retention times confirm the identity of each component. The absence of retention time shifts in the combined ZER and PDZ sample demonstrates that the

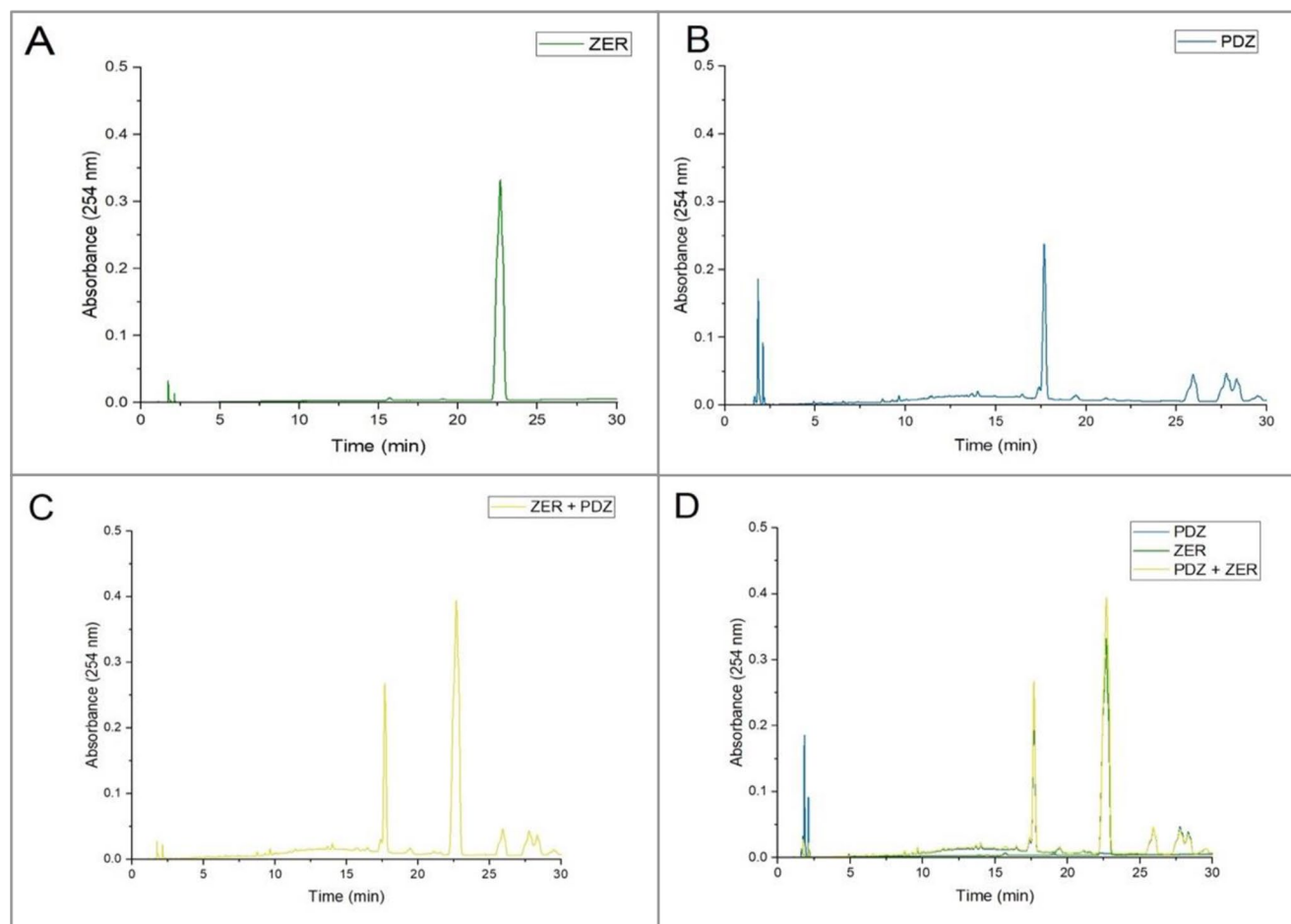


Fig. 6 RP-HPLC chromatogram of the ZER (A), PDZ (B), the ZER+PDZ mixture (C), and overlay of the chromatographic profiles (D) from A, B, and C

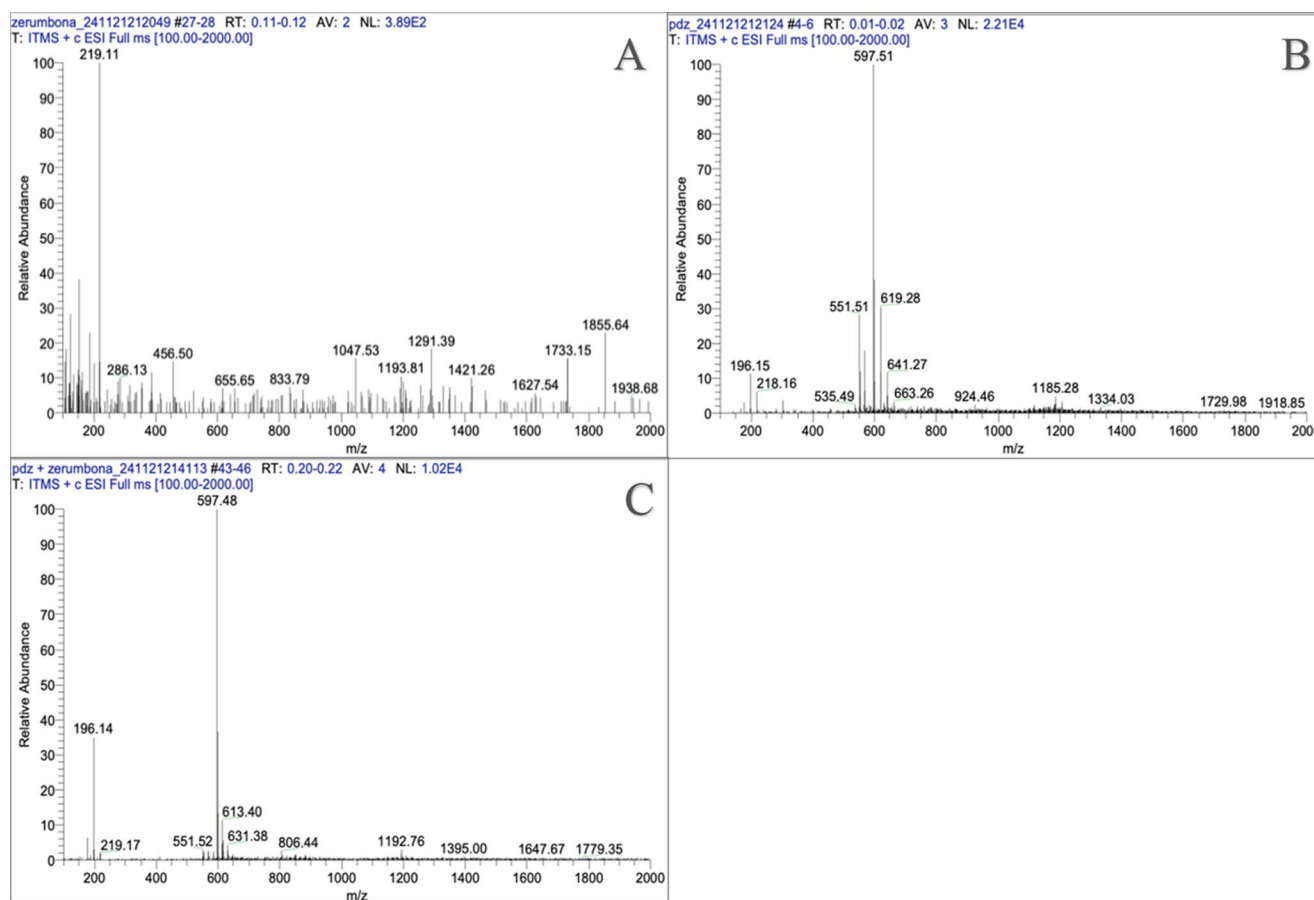


Fig. 7 Mass spectra obtained for ZER (**A**), PDZ (**B**) and ZER+PDZ (**C**). **A**: ZER's theoretical molar weight (MW)=218.33 g mol⁻¹, obtained $m/z=219.11$ ($Z=1$). **B**: PDZ's theoretical molar weight

(MW)=596.67 g mol⁻¹, obtained $m/z=597.51$ ($Z=1$). **C**: ZER+PDZ=obtained $m/z=597.48$ ($Z=1$)

chemical composition of neither ZER nor PDZ was altered by mixing.

Mass spectrometry

The identification of the ZER and PDZ molecules are detailed in Figs. 7A and B, respectively. When observing the molecules of ZER mixed with PDZ (Fig. 7C), no change in the chemical composition of PDZ was observed following the addition of ZER. The m/z value of 597.42 ($z=1$), characteristic of PDZ, remained consistent in the presence of ZER.

Discussion

The resistance to conventional drugs has posed a significant challenge to infection control, and aPDT has been proposed as an effective alternative to combat biofilm-associated infections. It has been reported that Gram-positive bacteria are more sensitive to the action of the photosensitizer and, consequently, to the effects of aPDT than Gram-negative

bacteria [28]. Differences in sensitivity of aPDT has been attributable to physiological distinctions between their cell walls. Gram-positive bacteria have a cytoplasmic membrane encased within a porous peptidoglycan layer, facilitating photosensitizer penetration. Gram-negative bacteria exhibit a more complex envelope, consisting of an inner cytoplasmic membrane and an outer membrane, separated by a peptidoglycan layer, which collectively presents a significant barrier to photosensitizer diffusion [17]. Furthermore, it was observed that fluconazole-resistant *C. albicans* also exhibits reduced susceptibility to photodynamic treatment [29]. Therefore, approaches that are able to reduce extracellular polymeric substances weaken the biofilm structure and make it more susceptible to the action of antimicrobial therapies are promising. Some compounds can be combined with aPDT to enhance this anti-biofilm efficiency [13, 14, 21, 25]. Therefore, this study investigated the effect of a mixture containing a substance that disrupts the biofilms' ECM (ZER) with a photosensitizer (PDZ) against *S. aureus*, *E. coli*, fluconazole susceptible and resistant *C. albicans* biofilms, aiming to enhance aPDT treatment effectiveness

and reduce the treatment time. The results demonstrated that the mixture of ZER and PDZ in the photodynamic treatment [MIX(ZER+PDZ)+LED group] exhibited similar anti-biofilm activity than ZER applied before PDZ [21], optimizing the treatment time.

The biofilms treated with MIX(ZER+PDZ)+LED demonstrated the greatest reduction in CFU/mL of 2.74, 2.89, 2.45 and 2.07 log₁₀ for *S. aureus*, *E. coli*, fluconazole susceptible and resistant *C. albicans* biofilms, respectively, compared to their respective control group. A previous study that corroborates with our finding showed that the application of ZER prior aPDT treatment promoted a similar reduction in colony counts of 2.43 and 2.04 log₁₀ of fluconazole susceptible and resistant *C. albicans* biofilms, respectively [21], however a longer application time was necessary to achieve the same results (1 h versus 40 min in this study). Fluconazole-susceptible and resistant *C. albicans* biofilms were included in this study to compare and validate the mixture of ZER with PDZ, as well as to confirm the reproducibility of the treatment.

A key distinction of this research was the evaluation of the aPDT effect on biofilms formed by both Gram-positive and Gram-negative bacterial strains. Photosensitizers from chlorin e6 family, such as PDZ, have molecular groups at their ends, which may be a relevant factor in their internalization by bacterial cells [30]. A previous study, using PDZ-mediated aPDT (PDZ at 0.1 mg/mL⁻¹ for 24 h of pre-incubation and a fluence of 50 J cm⁻²) reported a significant reduction in the viability of planktonic cultures of *S. aureus* but failed to achieve inactivation of *E. coli* [31]. Similarly, PDZ-mediated aPDT significantly inhibited *S. aureus* growth after 24 h of pre-incubation, but not after 15 min. In contrast, PDZ-mediated aPDT showed no significant reduction in *E. coli* growth at the same incubation time [32]. In a study that employed methylene blue to mediate aPDT (50 J cm⁻²), reductions in viable colonies were observed for *S. aureus* (1.03 log₁₀) and *E. coli* (0.80 log₁₀) biofilms [33]. Similarly, when toluidine blue was used as a photosensitizer, reduction of 1.0 log₁₀ and 0.80 log₁₀ were reported for *S. aureus* and *E. coli* biofilms, respectively [34]. In the present study when photodynamic treatment (PDZ+LED group) was performed, reductions of 0.77 log₁₀ and 1.13 log₁₀ were observed for *S. aureus* and *E. coli* biofilms, respectively. These findings suggest that ZER enhances the efficacy of aPDT in biofilms regardless of strain characteristics (Gram-positive or Gram-negative) once that the greatest reduction in CFU/mL of 2.74 and 2.89 log₁₀ for *S. aureus* and *E. coli* were observed.

Previous studies have shown that only ZER demonstrated effective antibacterial activity in different tests against a variety of microorganisms, particularly Gram-positive bacteria [35]. ZER showed dose-dependent effects as

an antimicrobial and antibiofilm agent against *C. albicans* (single species) and *S. aureus* (dual species) biofilms [35, 36]. ZER inhibited biofilm formation and hyphal growth of *C. albicans* [36]. The α , β -unsaturated carbonyl seems to be responsible for ZER's effect by causing cell membrane disruption, what can explain the mechanism of action against methicillin-resistant *S. aureus* (MRSA) [15]. ZER shows anti-MRSA effects by causing membrane depolarization, increasing its permeability, and then rupturing it, which leads to bacterial death [37]. ZER also shows its effects by inducing and regulating ROS formation through different metabolic pathways (NADPH, mitochondria, oxidase, xanthine oxidase, nitric oxide synthase, cyclooxygenase, and enzymes), which can contribute to a better microbial inactivation [38]. In a previous study it was observed that ZER reduced the microbial viability of fluconazole-susceptible *C. albicans* by 38,51% and of fluconazole-resistant *C. albicans* by 36,99% [19]. Regarding the quantification of biofilm components, ZER (256 μ g/mL) also demonstrated its effects by reducing total biomass (57%), insoluble biomass (45%), WSP content (65%), proteins (18%) and eDNA (78%) in fluconazole-susceptible *C. albicans* biofilms. In fluconazole-resistant *C. albicans* biofilms, the observed reductions were in insoluble biomass (13%), proteins (18%), WSP (65%), ASP (10%) and eDNA (23%) [19]. However, when ZER was combined with aPDT, the effects were enhanced, leading to a greater reduction in viable cell counts and biofilms' components for all evaluated strains (CaS, CaR, R14 and R70). The combined therapy (ZER+aPDT) resulted in a 2.01 log₁₀ reduction (30%) in CFU counts, along with significant decreases in key biofilm components, including total biomass (30%), insoluble biomass (33%), and total protein (15%). Regarding the ECM components, the combination of therapies reduced insoluble proteins (24%), WSP (68%), ASP (26%) and eDNA (60%), demonstrating the highlighted effects after combining them.

To the best of our knowledge, this is the first study to investigate the efficacy of ZER mixed with PDZ mediating the aPDT in biofilms of *E. coli* and *S. aureus*, two significant bacteria commonly found in oral biofilms [24, 30, 32]. Other compounds such as DNase I, previously employed as an ECM disrupting agent prior to aPDT treatment, yielded colony count reductions of 2.16 log₁₀ and 1.92 log₁₀ for fluconazole-susceptible and resistant *C. albicans*, respectively [13]. Additionally, a previous study that investigated the use of hydrogen peroxide (H₂O₂) prior to aPDT for the same purpose reported reductions of 0.90 log₁₀ and 1.09 log₁₀ for fluconazole-susceptible and resistant *C. albicans*, respectively [22]. These results demonstrate that the ZER's treatment either before the aPDT or mixed with PDZ achieved a greater reduction in CFU counts compared to other ECM disrupting agents, such as DNase I and H₂O₂.

Furthermore, ensuring the cytotoxic safety of these treatments in oral mammalian cells is crucial. Achieving a balance between a biomolecule's effectiveness and safety is typically a challenging effort [39]. Our results demonstrate no cytotoxicity activity of the evaluated treatments [ZER+PDZ+LED or MIX(ZER+PDZ)+LED] against NOK-si and FGH cells. The reduction in cell viability did not exceed 17%, which is in accordance with the acceptable limit of 30% [40]. Previous studies related that ZER is no cytotoxic in Vero mammalian cells, fibroblast cells and hepatic cells [41, 42]. However, it is important to note that mammalian cell susceptibility tends to be higher in MTT assays than in in vivo conditions due to direct cell exposure to biomolecules, without the influence of in vivo variables such as administration route and topical absorption, which can influence the in vitro cytotoxicity effect [42].

Combining substances can lead to interactions in which one substance affects how another substance works [43]. Since ZER is a compound with various molecular regions capable of reacting with other molecules, it is essential to perform analysis such as absorbance spectroscopy (AS), high-performance liquid chromatography (HPLC), and mass spectrometry (MS) to verify whether the mixture exhibits any interactions. The results indicate that there was no change in the chemical-molecular composition after mixing ZER with PDZ in a single working solution, as no statistical difference in absorbance was observed between PDZ and the ZER+PDZ mixture. The results show that there was also no change in the anti-biofilm effect of ZER and PDZ after the mixture in a single working solution. In addition, the mixture was not cytotoxic for the oral cells (FGH and NOK-si).

The absence of retention time shifts in the HPLC analysis of the ZER and PDZ mixture demonstrates that the chemical composition of both ZER and PDZ remained unaffected by mixing. The m/z value of PDZ remained consistent in the presence of ZER. The spectral bands observed in this study are also consistent with findings reported in previous investigations for either ZER or PDZ [44, 45]. In a study evaluating the synergistic effect of combining helical cationic antimicrobial peptides with aPDT against *Enterococcus faecalis*, it was observed that photosensitizers from the methylene blue and chlorin-e6 families, such as PDZ, were more compatible with new compounds without undergoing molecular changes [46]. The mixture of ZER+PDZ exhibited all the characteristic peaks corresponding to the functional groups of ZER and PDZ individually. Notably, no novel peaks emerged, nor were substantial shifts observed in the positions of existing peaks. These results suggest that the mixture of ZER and PDZ does not induce any significant alterations or indicate incompatibility between the two compounds for mediate the aPDT.

aPDT is a scientifically validated approach for the control of oral biofilms [47–49], and ZER has been shown to enhance its antibiofilm efficacy [21]. In the present study, the combination of ZER and PDZ MIX(ZER+PDZ)+LED demonstrated comparable antibiofilm effects to those observed when ZER was used before PDZ (ZER+PDZ+LED), with the added benefit of optimized application time. The of ZER+PDZ-mediated photodynamic treatment was effective against *E. coli*, *S. aureus*, fluconazole susceptible and resistant *C. albicans* indicating its potential as a promising therapeutic strategy. Further studies using in vitro mixed-species biofilms, in vivo models of infections models and randomized clinical trials are recommended to confirm these findings and assess the clinical applicability of this approach.

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

Declarations

Ethical approval Not applicable.

Competing interests The authors declare no competing interests.

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