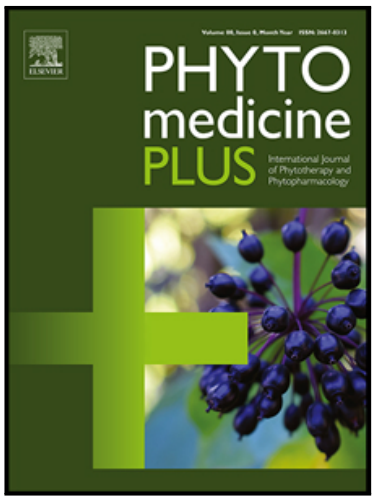

Enhanced photodynamic effect of a natural anthraquinone on Candida tropicalis biofilms using a stepwise irradiation strategy.

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PII: S2667-0313(24)00017-4
DOI: https://doi.org/10.1016/j.phyplu.2024.100539
Reference: PHYPLU 100539

To appear in: *Phytomedicine Plus*

Received date: 23 October 2023
Revised date: 30 January 2024
Accepted date: 19 February 2024

Please cite this article as: Juliana Marioni , María L. Mugas , Florencia Martinez , Bianca Romero , Tomás I. Gómez , Giuliana Lingua , Brenda Konigheim , Susana C. Núñez Montoya , Enhanced photodynamic effect of a natural anthraquinone on Candida tropicalis biofilms using a stepwise irradiation strategy., *Phytomedicine Plus* (2024), doi: https://doi.org/10.1016/j.phyplu.2024.100539

Enhanced photodynamic effect of a natural anthraquinone on *Candida tropicalis* biofilms using a stepwise irradiation strategy.

Juliana Marioni[a,b], María L. Mugas[a,b], Florencia Martinez[c,d], Bianca Romero[a,b], Tomás I. Gómez[a,b], Giuliana Lingua[c,d], Brenda Konigheim[c,d], Susana C. Núñez Montoya[a,b]*

[a]Instituto Multidisciplinario de Biología Vegetal (IMBIV), Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET). Córdoba, Argentina.

[b]Universidad Nacional de Córdoba, Facultad de Ciencias Químicas, Departamento de Ciencias Farmacéuticas. Edificio Ciencias 2, Ciudad Universitaria. Córdoba. Argentina.

[c]Universidad Nacional de Córdoba, Facultad de Ciencias Médicas, Instituto de Virología "Dr. J. M. Vanella". Córdoba, Argentina.

[d]CONICET. Córdoba, Argentina.

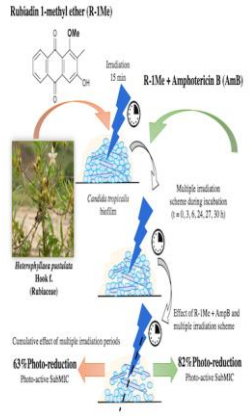
*Correspondence:

Prof. PhD, Susana C. Núñez Montoya, Departamento Ciencias Farmacéuticas, Facultad Ciencias Químicas, Universidad Nacional Córdoba, IMBIV-CONICET. Haya de la Torre y Medina Allende, X5000HUA Córdoba, Argentina.

Cel: +54 9 351 6162526

Email: sununez@unc.edu.ar

Graphical abstract



Abstract

Background: Antimicrobial Photodynamic Therapy (aPDT) has demonstrated effectiveness against various *Candida* biofilms, typical resistant to conventional treatments. Some strategies have shown to enhance the photoactivity of some photosensitizers (PS), such as the use of a multiple irradiation scheme or the combination with drugs that improve the penetration of the PS through the microbial membrane.

Purpose: Having demonstrated the photodynamic antibiofilm activity of some natural anthraquinones (AQs), we selected rubiadin 1-methyl ether (R-1ME) that showed a low photo-reduction percentage (%R) on the biofilm mass, with the aim to improve its effect.

Study Design: Experimental *in vitro* photo-stimulation protocols have been developed, which include successive light exposures and the combination of this AQ with a commonly used antifungal such as Amphotericin B (AmB).

Methods: The biofilms reduction was quantified by Crystal Violet staining. Reactive oxygen and nitrosative species were observed as action mechanism, alongside an assessment of antioxidant response through superoxide dismutase enzyme activation and total antioxidant system capacity.

Results: Applying R-1ME to *C. tropicalis* biofilms with sequential 15-min irradiation sessions at varied incubation intervals (0, 3, 6, 24, 27, and 30 h) yielded a substantial photo-reduction (62.9 %R) on biofilm mass, even halving the bioactive concentration of R-1ME. Moreover, combining R-1ME with AmB under this irradiation pattern produced an even greater impact (82 %R) at concentrations below the Minimal Inhibitory Concentration. Evident redox imbalances in the biofilm were linked to this photosensitized activity.

Conclusions: A new strategy was found to improve the activity of a natural PS on fungal biofilms, by combining it with antifungal drugs, under a staged irradiation scheme, which, in turn, required low doses of the PS and the antifungal to achieve this improved photo-reduction.

Keywords: anthraquinones, photodynamic activity, *Candida* biofilms, multiple irradiation steps, oxidative stress.

Abbreviations: AmB: amphotericin B, aPDT: antimicrobial Photodynamic Therapy, AQs: anthraquinones, BBU: Biofilm Biomass Unit, ^{13}C-NMR: carbon 13 nuclear magnetic resonance spectroscopy, CLSI: Clinical and Laboratory Standards Institute, CV: Crystal Violet, DMSO: dimethyl-sulfoxide, FRAP: Ferric Reducing Antioxidant Potency assay, HPLC: High Performance Liquid Chromatography, IR: Infrared spectroscopy, MIC: Minimal Inhibitory Concentration, MS: Mass Spectrometry, $^{3}O_2$: molecular oxygen, NBT: Nitro BlueTetrazolium, NCPF: National Collection of Pathogenic Fungi, NO•: nitric oxide, $O_2^{\bullet -}$: superoxide radical anion, OD: optical density, PBS: Phosphate Buffered Saline, pMIC: photoactive Minimal Inhibitory Concentration, PS: photosensitizers, pSubMIC: photoactive Sub Minimal Inhibitory

Concentration = pMIC/2, pSupraMIC: photoactive Supra Minimal Inhibitory Concentration = pMICx2, ^{1}H-NMR: proton nuclear magnetic resonance spectroscopy, R-1ME: rubiadin 1-methyl ether, RNI: reactive nitrogen intermediates, ROS: reactive oxygen species, %R: reduction percentage, SDB: Sabouraud Dextrose Broth, SOD: superoxide dismutase enzyme, SubMIC: Sub Minimal Inhibitory Concentration = MIC/2, SupraMIC: Supra Minimal Inhibitory Concentration = MICx2, UV–Vis: ultraviolet visible spectrophotometry.

INTRODUCTION

Over the past few decades, there has been a notable rise in opportunistic *Candida* infections. This increase can be attributed to several factors, including the growing number of immunosuppressed patients, the utilization of biomedical devices such as catheters and prostheses, the prevalence of the acquired immunodeficiency syndrome, and the emergence of resistance to antifungal drugs due to their misuse (Hossain et al., 2022). *Candida* infections have been identified as a major contributor to nosocomial infections (Atiencia-Carrera et al., 2022). These fungal infections are commonly associated with the formation of biofilms, which significantly reduce the effectiveness of antifungal agents and consequently lead to higher rates of morbidity and mortality (Atiencia-Carrera et al., 2022; Hossain et al., 2022). *Candida* species possess the capability to create dense biofilms, with *Candida albicans* being the most frequently isolated pathogen. However, other species of *Candida*, such as *Candida tropicalis*, are also frequently encountered in these infections (Sharma and Chakrabarti, 2023).

Given the inadequate effectiveness of conventional treatments for *Candida* infections, there is a pressing need to explore new therapeutic alternatives, particularly for infections caused by biofilm formation. One such promising approach is antimicrobial Photodynamic Therapy (aPDT), which has demonstrated effectiveness against various fungal biofilms (Rodríguez-Cerdeira et al., 2021). The aPDT involves the coordinated interaction of three key components: a non-toxic chemical compound known as a photosensitizer (PS), harmless light at its absorption wavelength, and molecular oxygen ($^3O_2$) present in the surrounding environment. When the PS is activated by light in the presence of $^3O_2$, generates an increase in levels of reactive oxygen species (ROS). Thus, oxidative stress is induced in

pathogenic microorganisms. The ROS generated during aPDT have the ability to react with biological molecules in the immediate environment, such as proteins, lipids, and deoxyribonucleic acid. This interaction can ultimately result in the death of the pathogenic microbial cells (Kolarikova et al., 2023; Rodriguez-Cerdeira et al., 2021).

Numerous approaches have been explored to enhance the effectiveness of aPDT (Kolarikova et al., 2023; Mariño-Ocampo et al., 2023; Torres-Hurtado et al., 2019; Vera et al., 2022). Among them is the utilization of a multiple irradiation scheme, where light exposure occurs at different time intervals during the incubation period, interspersed with periods of darkness. The rationale behind this approach is to allow for increased $^{3}O_2$ concentration during the dark intervals, which is expected to improve the response to aPDT (Figueredo et al., 2017; Torres-Hurtado et al., 2019). Previous research has indicated that multiple applications of aPDT on *C. glabrata* biofilms yield superior outcomes compared to single-session therapies. This phenomenon may be attributed to the weakening of the biofilm surface through successive irradiations, rendering it more susceptible to the treatment (Figueredo et al., 2017).

However, it is important to consider that the fungal cell wall poses an additional challenge in terms of permeability, owing to its composition. This moderately thick layer, consisting of β-glucan and chitin, creates resistance against drugs. Consequently, PS that possess the capability to penetrate the cell wall, along with agents that improve PS penetration, can significantly enhance the efficiency of the photosensitized effect (Ziental et al., 2021).

In a previous study, we demonstrated the photo-reduction of *C. tropicalis* biofilms using two natural photosensitizing anthraquinones (AQ): rubiadin (RB) and rubiadin 1-methyl ether (R-1ME) (Fig. 1). The treatment involved a single irradiation period prior to biofilm incubation. The observed effect was attributed to an oxidative and nitrosative imbalance. RB exhibited greater activity than R-1ME, as it resulted in a higher reduction of biofilm biomass, requiring a lower concentration (63.5 ± 4.5% reduction at 1.96 µg/mL) compared to R-1ME (47 ± 10% reduction at 15.6 µg/mL) (Marioni et al., 2016a). Furthermore, our findings indicated that R-1ME exhibited a higher accumulation rate within *C. tropicalis* biofilms compared to RB (Marioni et al., 2017), and less cytotoxicity than RB on Vero cells, a mammalian eukaryotic cell line widely used to assess such effects (Konigheim et al 2012).

Therefore, the objective of this study was to enhance the efficacy of R-1ME by implementing a multiple irradiation scheme, involving an increased number of irradiation periods, on *C. tropicalis* biofilms treated with R-1ME alone and in combination with amphotericin B (AmB), an antifungal drug that targets the cell membrane (Cavassin et al., 2021). Furthermore, we investigated the impact of this treatment on stress metabolites: including superoxide radical anion ($O_2^{\bullet -}$) and nitrites, as well as the activation of antioxidant systems within the biofilms, such as the superoxide dismutase enzyme (SOD) and total antioxidant capacity. By harnessing the oxidative stress induced by aPDT, the goal is to disrupt and eradicate the *Candida* biofilms, which are notoriously resistant to traditional treatments.

MATERIAL AND METHODS

Natural compound

R-1ME was isolated from aerial parts of *Heterophyllaea pustulata* Hook.f. (Rubiaceae). It was obtained with a purity of 93.8 ± 0.1 % as determined by High Performance Liquid Chromatography (HPLC) analysis (Marioni et al., 2016a; Marioni et al., 2017; Núñez Montoya et al., 2003). Its identity was unequivocally confirmed by the analysis of its spectroscopic/spectrometric data (^{1}H-NMR, ^{13}C-NMR, IR, UV–Vis, MS) in comparison with literature references (Núñez Montoya et al., 2003).

Sample preparation

The photoactive Minimal Inhibitory Concentrations (pMICs) of R-1ME and AmB were determined in the planktonic form of *C. tropicalis* yeasts and were used as a reference to establish the concentrations to be tested in biofilms by Marioni et al (2016a). Thus, the 3 concentrations tested for R-1ME were: the pMIC (15.6 µg/mL), a pSubMIC (pMIC/2 = 7.8 µg/mL), and a pSupraMIC (pMIC x 2 = 31.3 µg/mL); whereas for AmB were 0.25 µg/mL (SubMIC), 0.5 µg/mL (MIC) and 1 µg/mL (SupraMIC). A stock solution of R-1ME was prepared in Sabouraud Dextrose Broth (SDB) (Difco, Detroit, MI, USA) with 1% dimethyl-sulfoxide (DMSO) and subsequently diluted in SDB to obtain the final concentrations for testing.

Candida tropicalis biofilm formation

The standard strain *C. tropicalis* NCPF 3111 (National Collection of Pathogenic Fungi, Bristol, UK) was used to generate biofilms, which were preserved and reactivated in accordance with the guidelines provided by the Clinical and Laboratory Standards Institute (CLSI, 2002). Biofilm were formed in flat-bottomed 96-well microplates (Greiner Bio-One, Frickenhausen, Germany) following an adaptation of the method initially described by O'Toole and Kolter (Marioni et al., 2016b; O´Toole and Kolter, 1998). After 48 h of incubation at 37 ºC without shaking, the supernatant

was discarded, and the resulting biofilms were subjected to various treatments with R-1ME. The total biomass of the biofilms was quantified by Crystal Violet (CV) staining (1 % w/v for 5 min). Excess dye was removed with sterile Phosphate Buffered Saline (PBS) before extracting it from the biomass using a solution of ethanol/acetone (70:30). The optical density (OD) was measured spectrophotometrically at 595 nm using a microplate reader (Tecan Sunrise Model, TECAN, Grödig, Austria). Results were expressed in biofilm biomass unit (1 BBU = 0.1 DO595nm) (Marioni et al., 2016b).

Irradiation system

An actinic Phillips 20W lamp (380 ± 480 nm, 0.65 mWcm-2) with an emission maximum at 420 nm was used. It was placed inside a black box at 20 cm above the samples (Marioni et al., 2016b).

In vitro photo-reduction biofilm assay using a multiple scheme of irradiation.

Two microplates were simultaneously prepared; one was kept in darkness whereas the other was irradiated at different time points during the incubation period of the biofilms, both treated and untreated with R-1ME. The microplate was initially irradiated for 15 min immediately after the addition of the R-1ME solutions (t = 0 h). Subsequently, it was irradiated at 3, 6, 24, 27 and 30 h after the mentioned treatment, with each irradiation session lasting 15 min. This specific irradiation scheme was based on the results obtained from the HPLC accumulation assay of R-1ME in *C. tropicalis* biofilms, which indicated that the maximum accumulation of R-1ME in the biofilm occurs after 3 h of incubation (Marioni et al., 2017). Between each irradiation session, the microplates were incubated at 37 ºC until a total incubation period of 48 h was completed. Negative controls, SDB alone and SDB with 1%

DMSO, were included in the experiment. Following incubation, the supernatant from both microplates was collected to assess the production of $O_2^{\bullet -}$, reactive nitrogen intermediates (RNI) and the activation of the antioxidant system: SOD and total non-enzymatic system by means of the ferric reducing antioxidant potency assay (FRAP). Total biomass of the biofilm was quantified using CV staining, as described previously.

In vitro antifungal combination assay over biofilms applying a multiple scheme of irradiation.

Three working solutions of R-1ME were combined with 3 concentrations of AmB (MIC = 0.5 µg/mL, SubMIC = 0.25 µg/mL, and SupraMIC = 1 µg/mL). R-1ME solutions were added by rows, whereas AmB solutions were added by columns, simultaneously applied to a 48-h-old *C. tropicalis* biofilm (Dastgheyb et al., 2013; Marioni et al., 2016a). Like the previous assay, two microplates were prepared simultaneously. After the addition of R-1ME/AmB solutions, one microplate was kept in darkness, whereas the other was subjected to irradiation following the previously described procedure. Following incubation at 37 ºC for 48 h, the supernatants were collected to analyze the production of $O_2^{\bullet -}$ and RNI, as well as the activation of the enzymatic (SOD) and non-enzymatic antioxidant system (FRAP). The effect of combination treatment on the biofilm biomass was determined using CV staining and expressed as BBU. Additionally, the reduction percentage of the biofilm biomass was calculated by applying the following formula (Marioni et al., 2016a):

$$\%R = \frac{\text{Control } OD_{595nm} - \text{Sample } OD_{595nm}}{\text{Control } OD_{595nm}} \times 100$$

Assays for oxidative and nitrosative metabolites and antioxidant activity of biofilms

The production of $O_2^{\bullet -}$ and RNI, as well as the SOD activity and total antioxidant capacity of the biofilm, were measured in the supernatant following the photodynamic treatments on the biofilms.

The production of $O_2^{\bullet -}$ was assessed by using the nitro blue tetrazolium (NBT) reduction method (Mattila et al., 2015). The formation of blue diformazan is proportional to the generated $O_2^{\bullet -}$ in the biofilms, and its absorbance was measured spectrophotometrically at 540 nm. Results were expressed as OD_{540nm}/BBU ($O_2^{\bullet -}$/BBU) (Marioni et al., 2016a; 2016b, 2017).

Nitrosative stress was evaluated by measuring nitrite formation using the Griess reaction and a calibration curve of $NaNO_2$ as a standard (Tsikas, 2007). OD was measured at 540 nm (Marioni et al., 2016b; Tsikas, 2007), and the results were expressed as the ratio between nitrite concentration values generated per BBU (RNI/BBU).

SOD activity was detected by measuring the ability of this enzyme to inhibit NBT reduction in the presence of $O_2^{\bullet -}$, which is generated by the photoexcitation of riboflavin in the presence of oxygen and an electron donor (methionine). The results were expressed as SOD activation (%SOD/BBU) (Beauchamp and Fridovich, 1971; Marioni et al., 2016b).

The FRAP assay was performed following the procedure described by Benzie and Strain (1996). OD was measured at 593 nm, and the results were expressed as Fe^{+2} concentration values per BBU (FRAP/BBU) by using a $FeSO_4$ calibration curve (Marioni et al., 2016b).

Statistical analysis

All assays were performed in triplicate and repeated in 3 independent experiments. The data are presented as means ± standard deviation. A p value < 0.05 was considered statistically significant, determined by the t- Student-Newman-Keuls test for multiple comparisons. The symbol * denotes statistical significance at p < 0.05 when was compared to untreated biofilms, whereas the symbol # indicates statistical significance at p < 0.05 when comparing darkness and irradiation conditions.

RESULTS

Photo-reduction effect on *C. tropicalis* biofilm

The *in vitro* photo-reduction biofilm assay using a multiple scheme of irradiation established that the application of R-1ME under dark conditions did not have any impact on the biofilm biomass at all concentrations tested (green line in Fig. 2A). The reduction in biomass by photosensitization was demonstrated when the biofilm of *C. tropicalis* NCPF 3111 was treated with this AQ and exposed to irradiation once before incubation at pMIC (15.6 µg/mL, t = 0 h, red line in Fig. 2A). This effect was improved by the cumulative action of multiple irradiation periods, since R1-ME began to be active at a lower concentration (pSubMIC = 7.8 µg/mL) than pMIC (t = 0, 3, 6, 24, 27, 30 h, yellow line in Fig.2A); even, the %R was enhanced at the pMIC under this repeated irradiation scheme (62.9 ± 7.4 %R *vs.* 47 ± 10 %R). As a result, the photoactive concentration of the PS was halved (pSubMIC: 7.8 µg/mL). Note that when only one irradiation period (t = 0 h) was applied, R-1ME did not exhibit any photoactivity at pSubMIC in accordance with Marioni et al. (2016a). Contrary to expectations, with increasing AQ concentration, a loss of photoreduction of the biofilm was observed, specifically at the pSupraMIC for both irradiation experiments.

This could be due to the formation of molecular aggregates of the AQ, a characteristic of some PS, which ultimately results in low or no ROS production (Marioni et al., 2017). It is important to mention that the irradiation conditions alone did not have any effect on the biomass of the biofilm (data not shown).

In Figure 2B, *in vitro* antifungal combination assay of R-1ME with AmB over biofilms, applying a multiple scheme of irradiation, is depicted. The aim was to compare the effects of these combinations with the effects of each separate compound at their respective concentrations (yellow line: R-1ME, black line: AmB). Others plotted lines represent a specific concentration of AmB: blue line is SubMIC (0.25 µg/mL), purple line is MIC (0.5 µg/mL) and pink line is SupraMIC (1 µg/mL); which remained constant and was combined with each tested concentration of R-1ME (pSubMIC, pMIC, and pSupraMIC). When both drugs were combined at its pSubMIC (7.8 µg/mL for R-1ME and 0.25 µg/mL for AmB) under the irradiation scheme, it resulted in a significant photo-reduction (82 ± 2 %R) in the biomass of *C. tropicalis* biofilm. Thus, the utilization of AQ and AmB in combination with the multiple irradiation scheme not only enhanced the photo-reduction effect (82 ± 2 %R), also reduced the required concentration of both compounds by half.

Oxidative and nitrosative stress of biofilm and response of antioxidant mechanisms

The generation of $O_2^{\bullet -}$ and the production of nitrosative metabolites were investigated using the photoactive concentration of R-1ME (pSubMIC 7.8 µg/mL) under the applied irradiation scheme. It was observed that $O_2^{\bullet -}$ levels increased significantly compared to untreated biofilms (Fig. 3A). Furthermore, the combination of R-1ME (pSubMIC 7.8 µg/mL) and AmB (pSubMIC 0.25 µg/mL) exhibited even higher $O_2^{\bullet -}$ production than that observed when *C. tropicalis* was treated with AQ

alone and subjected to either a single irradiation period or consecutive periods of irradiation.

Regarding the RNI generation by this AQ in *C. tropicalis* biofilms under irradiation (Fig. 3B), the observed increase was consistent across all tested conditions. In darkness, RNI levels generated by R-1ME at pSubMIC (7.8 µg/mL) alone and in combination with AmB at pSubMIC (0.25 µg/mL), under one irradiation step or the irradiation scheme, were comparable to those of the untreated biofilm.

Furthermore, SOD activity and the total antioxidant capacity of the biofilm were stimulated under all experimental irradiation conditions (Fig. 4A and B). When comparing the AQ alone at pSubMIC (7.8 µg/mL), its ability to activate SOD and the antioxidant system was lower under the irradiation scheme compared to when a single irradiation period was applied at pMIC (15.6 µg/mL, t = 0 h). Notably, the combination of R-1Me and AmB both at pSubMIC concentrations (7.8 µg/mL and 0.25 µg/mL, respectively) under the irradiation scheme resulted in the highest increase in the antioxidant system (enzymatic and non-enzymatic). The stimulation of SOD was directly proportional to the levels of $O_2^{\bullet -}$ observed for each treatment (Fig. 3A).

DISCUSSION

R-1ME exhibited a moderate antibiofilm effect on *C. tropicalis* NCPF 3111 when it was exposed to immediate irradiation after its application and prior to incubation (t = 0), resulting in a photo-reduction of 47 ± 10% at the pMIC (15.6 µg/mL) (Marioni et al., 2016a). To enhance this effect, it was proposed to increase the photo-reduction percentage by increasing the number of irradiation sessions. Considering that a 15-

min irradiation period did not affect the biofilm biomass, it was decided to maintain the same time intervals for irradiation while repeating the session at different time points during incubation. The decision to design the irradiation scheme was based on the observation that R-1ME reaches its maximum accumulation in the biofilm after 3 h of incubation (Marioni et al., 2017). By increasing the frequency of irradiation during the incubation period, the photo-active concentration of R-1ME was halved (pSubMIC 7.8 µg/mL). This optimized irradiation scheme not only allowed the use of a low concentration (pSubMIC) of R-1ME, but also significantly improved the photo-reduction of *C. tropicalis* biofilms (62.9 ± 7.4 %R) compared to a single irradiation session before incubation (Marioni et al., 2016a).

Hence, it can be inferred that the ability of R-1ME to accumulate within biofilms significantly enhances its photo-stimulated antibiofilm effect, which holds promising clinical implications. Similar findings have been reported for curcumin, another natural PS, where the highest inactivation of biofilms formed by *C. albicans*, *C. glabrata*, and *C. dubliniensis* was observed after 20 min of irradiation, a time necessary for the PS to accumulate within the biofilms (Andrade et al., 2013). Furthermore, in the case of Photodiazine®, multiple irradiation sessions (LED light) resulted in greater photo-reduction of cell viability and overall biomass in multispecies biofilms compared to a single irradiation session (Quishida et al., 2015). These findings highlight the importance of optimizing the timing and frequency of irradiation to maximize the antibiofilm efficacy of photosensitizers, thereby improving the outcomes of biofilm-related infections.

One of the significant effects of photodynamic inactivation is its ability to modify the biofilm structure, resulting in a reduction of the biomass (Quishida et al., 2015). Based on our previous findings that R-1ME was able to alter the biofilm structure

with a single irradiation session (Marioni et al., 2017), our current results indicate that consecutive applications of multiple irradiation periods could further enhance the disruption of the biofilm structure, which would render the biofilm more susceptible to the action of the PS.

Furthermore, the combination of AQ and AmB both at pSubMIC concentrations (7.8 µg/mL and 0.25 µg/mL, respectively) with the multiple irradiation scheme improved the photo-reduction (82 ± 2 %R) and halved the active concentration of both compounds.

The application of a multiple irradiation scheme on *C. tropicalis* biofilms treated with R-1ME and the R-1ME/AmB combination resulted in a predominant nitrosative stress over oxidative stress. This would be attributed to the high levels of $O_2^{\bullet -}$, which induced the generation of nitric oxide (NO•) as a ROS scavenger (Kwun and Lee, 2020; Zhang et al., 2021). It is important to note that these measurements were taken after 48 h of biofilm incubation, and it is likely that the generated $O_2^{\bullet -}$ was counteracted by various mechanisms. Consequently, along with the deactivation by NO•, enzymatic antioxidant mechanisms such as SOD, as well as the total antioxidant system (FRAP), could contribute to minimize the levels of this ROS. Hence, the enhanced photo-reduction effect observed in *C. tropicalis* biofilms treated with R-1ME alone at pSubMIC (7.8 µg/mL) and in combination with AmB at pSubMIC (0.25 µg/mL) under the irradiation scheme may be attributed to the increased production of RNI compared to the effect of this AQ with a single irradiation period.

There are several mechanisms proposed to explain the enhanced effect observed when two compounds are combined. The potentiation of effect observed with the R-1ME-AmB combination can be attributed to the increased penetration of R-1ME into

fungal cells, facilitated by the ability of AmB to generate pores in the cellular membrane (Cavassin et al., 2021). This disruption in the membrane allows a greatest access of R-1ME inside the cells, thereby exerting its action more effectively. Consequently, the combined effect could be explained by the distinct mechanisms of action exhibited by each compound individually (Jhonson et al., 2004). Simultaneous inhibition of multiple targets within fungal cells could be achieved, since AmB acts on the cellular membrane, whereas the antibiofilm effect of R-1ME is mediated by the Type I photosensitizing mechanism, resulting in oxidative stress accompanied by generalized nitrosative stress (Cavassin et al., 2021; Marioni et al., 2016a; 2016b).

Furthermore, it has been demonstrated that the activity of AmB is closely linked to the accumulation of ROS within fungal cells (Cavassin et al., 2021; Mesa-Arango et al., 2014). Therefore, AmB would contribute to the disruption of the defense system against oxidative stress generated in fungal cells when is combined with R-1ME. This combination would lead to an increased production of ROS, consequently enhancing the activity against biofilms. It is worth noting that another natural compound, curcumin, has also exhibited synergistic effects against *C. albicans* when was combined with AmB and fluconazole. This synergistic effect has been also associated with ROS generation (Sharma et al., 2010). Similarly, the combination of eugenol and AmB has shown greater antifungal activity against *C. albicans* biofilms compared to treatments with each individual compound. The induction of cell damage and death in this combination is attributed to the production of ROS, even using lesser toxic doses of AmB (Khan et al., 2019).

CONCLUSIONS

The utilization of a multiple irradiation scheme enhances the activity of R-1ME both as an individual treatment and in combination with AmB against *C. tropicalis* biofilms. Notably, R-1ME exhibits activity at lower concentrations that were previously ineffective when using only a single irradiation session. Furthermore, this approach allows for a reduction in the required concentration of AmB, providing an advantage by enabling its use at levels that do not induce adverse or toxic effects.

The improved photo-reduction effect can be also attributed to the ability of R-1ME to accumulate within the biofilm. As a result, it generates a significant oxidative and nitrosative imbalance within the biofilm when it was stimulated by light. This imbalance disrupts the biofilm's structure, leading to a reduction in its biomass.

Overall, aPDT offers a promising alternative for combatting *Candida* infections by specifically targeting biofilms and leveraging the destructive power of oxidative and nitrosative stress to eliminate pathogenic microorganisms. The combination of PSs with antifungal drugs enhances the effect since it allows the action of different mechanisms. The staged irradiation regimen enables substantial photo-reduction with minimal doses of PS alone or in combination with antifungal.

ACKNOWLEDGEMENTS

This work has been supported by: SECyT (Consolidar, tipo 2, s/res. N° 411/18 y 155/22), FONCyT (PICT 2018 Nº 4576, s/ res. ANPCyT n° 401/19), and CONICET (PIP 2021-2023, s/ res. 1639/2021). G Lingua, Tomás I. Gómez and Bianca Romero are doctoral fellow of CONICET. F. Martinez and M. L. Mugas are pos-doctoral fellow

of CONICET. J. Marioni, B. S. Konigheim and S. C. Núñez Montoya are members of the Research Career of CONICET.

AUTHORS' CONTRIBUTION

Term, conceptualization, and methodology: S.C. Nuñez Montoya and J. Marioni. Data collection: J. Marioni, M L. Mugas, B. Romero, T.I. Gomez, and G. Lingua. Analysis and interpretation of the data: J. Marioni, and S.C. Nuñez Montoya. Statical analysis: F. Martínez, and B.S. Konigheim. Drafting the manuscript: J. Marioni, and S.C. Nuñez Montoya. Critical revision: B.S. Konigheim, and S.C. Nuñez Montoya. Resources, supervision, project administration and funding acquisition: S.C. Nuñez Montoya.

CONFLICT OF INTEREST

Authors declare no conflict-of-Interest.

SUPPLEMENTARY MATERIALS

S1 can be found at DOI: 10.1016/j.phymed.2016.07.008

S2 Summary of photo-reduction effect of R-1ME on *C. tropicalis* NCPF 3111 biofilm, BBU values.

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work the authors used GPT-Chat to improve the English. After using this tool/service, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

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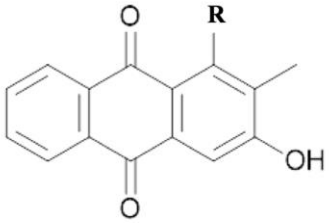

Anthraquinone	R
Rubiadin	OH
Rubiadin 1-methyl ether	OCH_3

Figure 1 Chemical structures of anthraquinones rubiadin and rubiadin-1-methyl ether

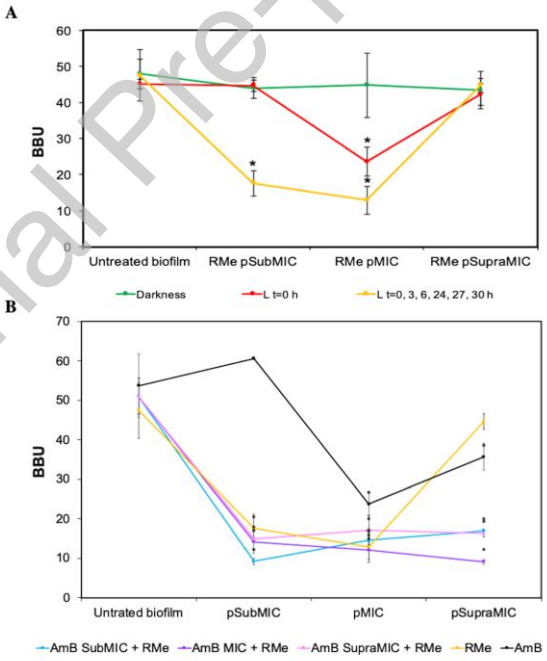

Figure 2 Photo-reduction effect of R-1ME on *Candida tropicalis* NCPF 3111 biofilm. (A) R-1ME effect with the application of a single irradiation period (t=0, red line) and with multiple irradiation periods (t = 0, 3, 6, 24, 27, 30 h, orange line). (B) Effect of R-1ME combined with AmB at different concentrations and the application of a multiple

irradiation scheme. AmB alone: green line, R-1ME alone: yellow line. *p<0.05 compared to untreated biofilm

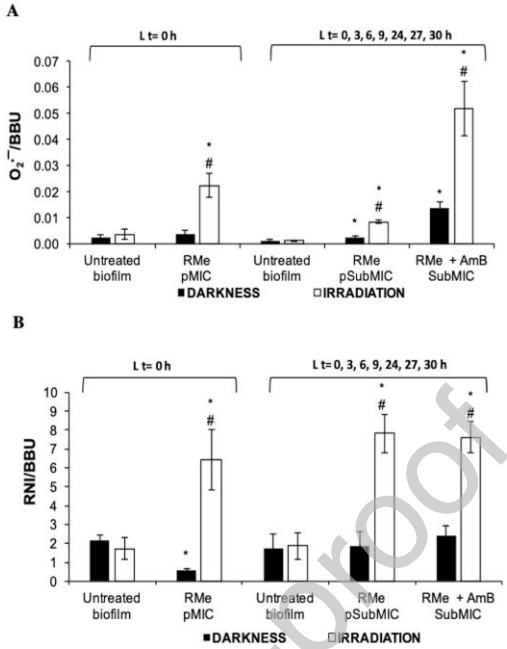

Figure 3 (A) ROS generation and (B) RNI production by R-1ME alone under a single irradiation period and the application of a multiple irradiation scheme on R-1ME alone and combined with AmB. *p<0.05 compared to untreated biofilm.

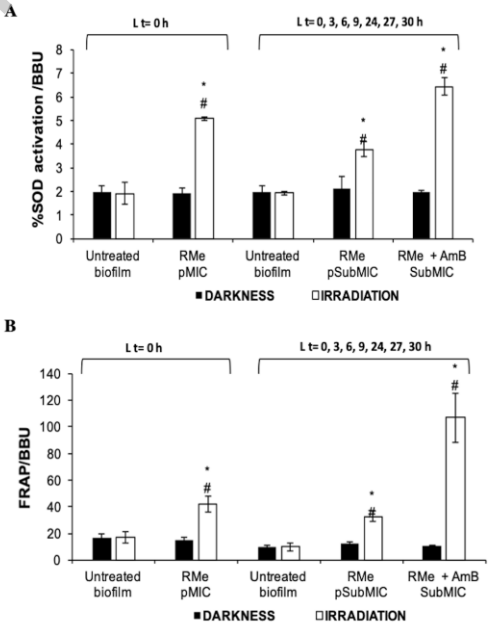

Figure 4 (A) SOD activation and (B) Total antioxidant capacity activation by R-1ME alone under a single irradiation period and the application of a multiple irradiation scheme on R-1ME alone and in combination with AmB.

AUTHOR AGREEMENT

We wish to confirm that the final version of the manuscript [**"Enhanced photodynamic effect of a natural anthraquinone on *Candida tropicalis* biofilms using a stepwise irradiation strategy"**] has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship and are not listed. We further confirm that the order of authors listed in the manuscript has been approved by all of us. We confirm that we have given due consideration to the protection of intellectual property associated with this work and that there are no impediments to publication, including the timing of publication, about intellectual property. In so doing we confirm that we have followed the regulations of our institutions concerning intellectual property.

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Signed by all authors as follows:

MARIONI Juliana 

MUGAS María Laura 

MARTINEZ Florencia 

ROMERO Bianca Carolina 

GOMEZ Tomás Isaac 

Giuliana, LINGUA 

KONIGHEIM Brenda Salomé 

NUÑEZ MONTOYA Susana Carolina 

Declaration of interests

☒ 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.

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Signed by all authors as follows:

MARIONI Juliana 

MUGAS María Laura

MARTINEZ Florencia 

ROMERO Bianca Carolina 

GOMEZ Tomás Isaac 

Giuliana, LINGUA 

KONIGHEIM Brenda Salomé 

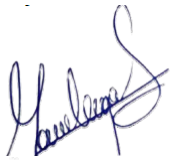
NUÑEZ MONTOYA Susana Carolina 

CONFLICT OF INTERESTS

The authors declare no conflict-of-Interest for this paper [**"Enhanced photodynamic effect of a natural anthraquinone on *Candida tropicalis* biofilms using a stepwise irradiation strategy"**].

Signed by all authors as follows:

MARIONI Juliana 

MUGAS María Laura 

MARTINEZ Florencia 

ROMERO Bianca Carolina

GOMEZ Tomás Isaac

Giuliana, LINGUA

KONIGHEIM Brenda Salomé

NUÑEZ MONTOYA Susana Carolina