2-Hydroxychalcone as a Potent Compound and Photosensitizer Against Dermatophyte Biofilms

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Dermatophytes, fungi that cause dermatophytosis, can invade keratinized tissues in humans and animals. The biofilm-forming ability of these fungi was described recently, and it may be correlated with the long treatment period and common recurrences of this mycosis. In this study, we evaluated the anti-dermatophytic and anti-biofilm activity of 2-hydroxychalcone (2-chalcone) in the dark and photodynamic therapy (PDT)-mediated and to determine its mechanism of action. Trichophyton rubrum and Trichophyton mentagrophytes strains were used in the study. The antifungal susceptibility test of planktonic cells, early-stage biofilms, and mature biofilms were performed using colorimetric methods. Topographies were visualized by scanning electron microscopy (SEM). Human skin keratinocyte (HaCat) monolayers were also used in the cytotoxicity assays. The mechanisms of action of 2-chalcone in the dark and under photoexcitation were investigated using confocal microscopy and the quantification of ergosterol, reactive oxygen species (ROS), and death induction by apoptosis/necrosis. All strains, in the planktonic form, were inhibited after treatment with 2-chalcone (minimum inhibitory concentration (MIC) = 7.8-15.6 mg/L), terbinafine (TRB) (MIC = 0.008-0.03 mg/L), and fluconazole (FLZ) (1-512 mg/L). Early-stage biofilms and mature biofilms were inhibited by 2-chalcone at concentrations of 15.6 mg/L and 31.2 mg/L in all tested strains. However, mature biofilms were resistant to all the antifungal drugs tested. When planktonic cells and biofilms (early-stage and mature) were treated with 2-chalcone-mediated PDT, the inhibitory concentrations were reduced by four times (2-7.8 mg/L). SEM images of biofilms treated with 2-chalcone showed cell wall collapse, resulting from a probable extravasation of cytoplasmic content. The toxicity of 2-chalcone in HaCat cells showed higher IC50 values in the dark than under photoexcitation. Further, 2-chalcone targets ergosterol in the cell and promotes the generation of ROS, resulting in cell death by apoptosis and necrosis. Overall, 2-chalcone-mediated PDT is a promising and safe drug candidate against dermatophytes, particularly in anti-biofilm treatment.

Keywords: dermatophytes, biofilms, 2-chalcone, photodynamic therapy, mechanism of action, Trichophyton rubrum, T. mentagrophytes
INTRODUCTION

Dermatophytes are filamentous fungi that may infect keratinized structures such as the skin, hair, and nails of humans and animals, causing dermatophytosis (Costa-Orlandi et al., 2014; Heidrich et al., 2015; Maraki and Mavromanolaki, 2016). This disease is globally considered as the most common dermatological zoonosis, with a prevalence of 20–25% in the global human population (Ivaskiene et al., 2016).

Phylogenetic re-taxonomy has been proposed for dermatophytes, suggesting their division into six genera (Zhan and Liu, 2017) and into nine genera (De Hoog et al., 2017). Nevertheless, the principal genera include Trichophyton, Microsporum, and Epidermophyton (Moriello, 2004; Costa-Orlandi et al., 2014; Maraki and Mavromanolaki, 2016; De Hoog et al., 2017). Dermatophytes can be grouped as anthropophilic, zoophilic, and geophilic (Moriello, 2004; Ivaskiene et al., 2016). T. rubrum is the most prevalent dermatophyte species, accounting for more than 80% of all infections. This species is commonly isolated in cases of tinea unguium, tinea corporis, tinea cruris, and tinea pedis (Faway et al., 2016; Zhan and Liu, 2017).

One of the main virulence factors of fungal species is the formation of communities called biofilms (Costa-Orlandi et al., 2017). In vitro biofilm formation by two of the most prevalent dermatophyte species, T. rubrum and T. mentagrophytes, was described (Costa-Orlandi et al., 2014) and was soon demonstrated in Microsporum canis (Danielli et al., 2017). In vivo biofilm formation by dermatophytes in patients with dermatophytosis is currently under investigation. However, a hypothetical relationship has been reported between biofilm formation and the persistent clinical condition of onychomycosis infections that have firm white masses of adhesion and are challenging to remove and treat (Burkhart; Burkhart; Gupta, 2002; Gupta, Daigle, Carvıl, 2016; Gupta and Foley, 2019).

Although a reasonable number of antifungal drugs are available for treating dermatophytosis, there has been little progress in new drug development in the last two decades, and recurrences in onychomycosis cases have increased by 50% (Gupta, Daigle, et al., 2016; Zhan and Liu, 2017). Most of these drugs are currently under investigation. However, a hypothetical relationship has been reported between biofilm formation and the persistent clinical condition of onychomycosis infections that have firm white masses of adhesion and are challenging to remove and treat (Burkhart; Burkhart; Gupta, 2002; Gupta, Daigle, Carvıl, 2016; Gupta and Foley, 2019).

Although a reasonable number of antifungal drugs are available for treating dermatophytosis, there has been little progress in new drug development in the last two decades, and recurrences in onychomycosis cases have increased by 50% (Gupta, Daigle, Carvıl, 2016; Singh et al., 2018a). Most of these drugs are classified into two families: azoles and allylamines (Gupta and Cooper, 2008; Aggarwal and Goindi, 2012). Azole derivatives, other than terbinafine and naftifine, are commonly prescribed to topically treat superficial infections in the early stages (Makimura et al., 1999; Gupta and Cooper, 2008; Aggarwal and Goindi, 2012). In extensive infections, chronic infections, or onychomycosis, oral drugs such as terbinafine, itraconazole, fluconazole, griseofulvin, and ketoconazole are used (Gupta and Cooper, 2008; Aggarwal and Goindi, 2012; Aggarwal et al., 2020). Due to the long treatment period for these infections, coupled with the considerable toxicity of most oral antifungal agents, monitoring is necessary for the hepatic, renal, and hematopoietic functions (Gupta and Cooper, 2008; Gupta, Versteeg, Shear, 2017a).

Difficult eradication, drug toxicity, and high recurrence rate have encouraged the search for therapeutic alternatives for dermatophytosis, mainly if they are associated with biofilm formation. The anti-dermatophyte effects of essential oils from plants (Mahboubi; Heidarytabar; Mahdizadeh, 2017), plant extracts (Mahboubi; Kazempour, 2015; Sun et al., 2017), ozone gas, ozonized oil (Ouf et al., 2016), and photodynamic inactivation (Paz-Cristobal et al., 2014; De Oliveira et al., 2015; Shamali et al., 2018), have already been described.

Chalcones or 1,3-diphenyl-2-propan-1-one are polyhydroxy compound precursors for flavonoids and iso flavonoids, which are abundant in fruits, vegetables, and edible plants (Gupta; Jain, 2015; Fu et al., 2016; Karimi-Sales; Mohaddes; Alipour, 2017). However, these compounds can be synthesized in the laboratory using the Claisen–Schmidt condensation method (Karimi-Sales; Mohaddes; Alipour, 2017). Chalcones demonstrate many biological activities such as anti-inflammatory, anti-tumor (Khanapure et al., 2018), antioxidant (Singh et al., 2018b), anti-diabetic (Cai et al., 2017), antibacterial (Singh et al., 2018a), antifungal (Gupta; Jain, 2015; Fu et al., 2016; Illicachi et al., 2017), antiprotozoal (Illicachi et al., 2017; Tajuddeen et al., 2018), and antiparasitic (Kotlyar et al., 2019), as well as activities against Alzheimer’s disease (Zhang et al., 2018b) and cholinesterase inhibition (Shah et al., 2018) among others. Chalcones can be excellent photosensitizers for PDT. For instance, photodynamic inactivation has been demonstrated in Histoplasma capsulatum (Melo et al., 2017).

PDT is a non-invasive treatment comprising the use of laser light or light-emitting diode (LED) of a specific wavelength that activates a photosensitive agent in the presence of oxygen, which results in the production of reactive oxygen species (ROS), free radicals, and consequently, cell death (Davies et al., 2016; De Figueiredo Freitas et al., 2017; Yang et al., 2018; Yuan et al., 2017). PDT is a promising modality as it is effective against a wide range of microorganism species (De Figueiredo Freitas et al., 2017). Photodynamic inactivation of microorganisms can occur in strains that are susceptible or resistant to conventional drugs (Mai et al., 2017). Therefore, this study aimed to evaluate the efficacy of 2-chalcone in the dark and of 2-chalcone-mediated PDT against dermatophyte biofilms, as well as to investigate its toxicity and mechanism of action in human skin keratinocytes (HaCat).

MATERIALS AND METHODS

Microorganisms and Synthesis of 2-Chalcone

T. rubrum ATCC 28189, ATCC MYA-4438, and T. mentagrophytes ATCC 11481 were used in this study. All strains were obtained from the Clinical Mycology Laboratory of the Department of Clinical Analysis, Faculty of Pharmaceutical Sciences-UNESP, Brazil. Microorganisms were grown on malt extract agar [malt extract (Kasvi): 2%, peptone from animal tissue (Sigma-Aldrich): 2%, glucose (Synth): 2% and agar (Kasvi): 2%], pH 5.7, incubated at 28°C for 7 days or until sporulation (Garcia et al., 2020).

Synthesis of 2-chalcone was performed as described by Melo et al. (2017) in collaboration with Prof. Dr. Luís Octávio Regasini’s research group from the Institute of Biosciences, Letters and Exact Sciences-UNESP, Brazil. The compound was
In Vitro Susceptibility of Dermatophytes to 2-Chalcone and Antifungal Drugs and Determination of Minimum Fungicide Concentration (MFC)

Determination of the Minimum Inhibitory Concentration (MIC)

Susceptibility tests were performed according to the document M38-A2, proposed by the Clinical Laboratory Standards Institute (CLSI) (2008), with a minor modification of addition of resazurin (Costa-Orlandi et al., 2020). Briefly, 2-chalcone was solubilized in 100% dimethyl sulfoxide (DMSO) at a stock concentration of 30,000 mg/L and was stored at −80°C. The working solutions of 2-chalcone at concentrations ranging from 0.12 to 62.5 mg/L were prepared in Roswell Park Memorial Institute (RPMI)-1640 medium with L-glutamine, without sodium bicarbonate, and with phenol red as the pH indicator ( Gibco®), and buffered with 4-Morpholinepropanesulfonic acid hemisodium salt (MOPS) (Sigma-Aldrich), pH = 7. TRB (Sigma-Aldrich) (0.5–0.001 mg/L), FLZ (Sigma-Aldrich) (0.12–64 mg/L), and the T. rubrum ATCC MYA-4438 were also used for quality control. Fungal suspensions were prepared in 0.85% NaCl and conidia were adjusted to a final concentration of 2.5 × 10^5 cells/mL using a hemocytometer, before adding to microdilution plates. Dilutions of the compound and antifungal drugs were dispensed in a 96-well microplate (Kasvi) at a total volume of 100 µL/well, following 100 µL of the inoculum. Visual and colorimetric readings were administered dose was 150 J/cm². The photosensitization assay was performed as described by Bile et al. (2020), with minor modifications. Briefly, biofilms were prepared as previously described, without phenol red in RPMI-1640 medium. Then, early-stage and mature biofilms were washed with sterile PBS and placed in contact with different concentrations of 2-chalcone (0.25–125 mg/L). The plates were incubated for 10 minutes in the dark and at 25°C and were then irradiated. After irradiation, the plates were incubated at 37°C for 96 hours. The analysis of the metabolic activity of cells was done by the XTT reduction assay.

Topographic Analysis of Biofilms by Scanning Electron Microscopy (SEM)

Early-stage and mature biofilms treated with 2-chalcone were processed as described by Martinez et al. (2010) and Costa-Orlandi et al. (2014). Biofilms were formed in 24-well plates, washed three times with PBS, and fixed with 800 µL of 2.5% glutaraldehyde solution (Sigma-Aldrich) for 1 hour at 4°C. The samples were then dehydrated with increasing ethyl alcohol concentrations (50–100%) at 25°C and were subsequently dried under the same conditions. Before microscopic analysis, the plate’s bottom containing the samples was cut with a scalpel, mounted on aluminum cylinders with silver (stubs), and placed on a high vacuum evaporator (Denton Vacuum Desk V, Jeol USA) for gold plating. The damage to the biofilm topography was analyzed using a scanning electron microscope Jeol JSM-6610LV at the School of Dentistry, UNESP-Araraquara, Brazil.
Cytotoxicity Assay for 2-Chalcone in Human Skin Keratinocytes (HaCat)
The cytotoxicity assay aimed to verify the selectivity index (SI) after the treatment of planktonic cells and mature biofilms with 2-chalcone in the dark and 2-chalcone-mediated PDT. The assay was performed as described by Costa-Orlandi et al. (2020) with minor modifications. Briefly, HaCat cells (CLS Cell Lines Service, 300493) were maintained in cell culture bottles with Dulbecco’s modified eagle’s medium (DMEM), containing 10% fetal bovine serum without phenol red, and incubated under standard conditions (37°C, 5% CO2). Cell suspensions were prepared to obtain a final concentration of 2 × 10⁶ cells/well in a 96-well microdilution plate. After 24 hours of incubation, the culture medium was removed and 200 µL of different concentrations of 2-chalcone were added. On some plates, photosensitization of 2-chalcone at 150 J/cm² was performed, and the others were kept in the dark. All the plates were incubated for 72 hours in the same conditions and protected from light. After incubation, 20 µL of resazurin (Sigma-Aldrich) at 60 µM was added and the plates were further incubated for 8 hours. Cell viability was assessed based on spectrophotometric (Epoch, Biotek) analysis at wavelengths 570 nm and 600 nm.

Determination of the Mechanism of Action Laser Scanning Confocal Microscopy
Confocal microscopy was used to evaluate the damage to the cell wall caused by 2-chalcone, using fluorochrome Calcofluor White (Thermo Fisher Scientific) and T. rubrum ATCC 28189. Fungal suspensions were prepared at a concentration of 1 × 10⁶ cells/mL and added to 24-well plates containing sterile coverslips, along with sub-inhibitory doses of 2-chalcone (7.8 mg/L). After the incubation period (35°C, for 96 hours), the supernatant was removed and the coverslips were washed with PBS. The coverslips were covered with Calcofluor White solution (100 mg/L), and the plates were incubated again at 37°C, for 45 minutes, protected from light. Then, the coverslips were washed with PBS, removed from the wells, and mounted on 4 µL of Fluoromount-G (Sigma-Aldrich), which was previously deposited on microscopic slides. The slides were then observed using a confocal microscope (Carl Zeiss LSM 800 with Airyscan) with an image capture and processing program (Software ZEN BLUE 2.3 System) at the Faculty of Dentistry, UNESP-Araraquara, Brazil (Curcio et al., 2017; Oliveira et al., 2020).

Quantification of Membrane Ergosterol
Ergosterol quantification was performed as described by Arthington-Skaggs et al. (1999) with minor modifications. Briefly, sub-inhibitory concentrations of 2-chalcone in the dark (7.8 mg/L), 2-chalcone-mediated PDT (1 mg/L), FLZ (128 mg/L), and amphotericin B (AMB) (1 mg/L) were used to treat a suspension of T. rubrum ATCC 28189 at a final concentration of 1 × 10⁶ cells/mL (diluted in RPMI-1640). The samples were incubated at 35°C, under agitation at 150 rpm for five days. After incubation, the samples were centrifuged and washed with sterile distilled water, and 3 ml of 25% alcoholic KOH solution was added. For sterol extraction, the samples were incubated in a water bath at 85°C for 1 hour after being transferred to glass tubes with a screw cap. The samples were cooled at 25°C, mixed with 1 mL of sterile distilled water, 3 mL of n-heptane, and sterile glass beads, followed by homogenization for 10 minutes on a vortex. The resulting supernatant (n-heptane layer) was transferred to microtubes and incubated at −20°C for 24 hours followed by an analysis on a visible UV spectrophotometer at a wavelength of 281 nm. Standard curves were prepared using 95% pure ergosterol at concentrations ranging from 75 to 10 mg/L.

Apoptosis/Necrosis Assay
The death mechanism in T. rubrum ATCC 28189 was studied after treatment with 2-chalcone in the dark, 2-chalcone-mediated PDT, AMB, and FLZ. Inocula were prepared and adjusted to a final concentration of 1 × 10⁶ cells/mL in a volume of 1.5 mL. The cells were treated with the same volume of compounds and AMB at a concentration of 4x MIC. FLZ treatment was performed at a concentration of 256 mg/L (as this strain was found to be resistant to FLZ). Cell death was evaluated using the Annexin V-FITC apoptosis detection kit (Sigma-Aldrich, A9210) following the manufacturer’s guidelines. The samples were analyzed on a BD FACS Canto I flow cytometer located at the Clinical Mycology Laboratory at the School of Pharmaceutical Sciences, UNESP-Araraquara, Brazil.

Quantification of ROS
Intracellular ROS production after treating T. rubrum ATCC 28189 with 2-chalcone in the dark and 2-chalcone mediated PDT was evaluated using 50 µM H2DCFDA (2.7 dichlorodihydrofluorescein diacetate, Invitrogen) as described by Singulani et al. (2019). This compound is converted to a highly fluorescent 2′,7’-dichlorofluorescein (DCF) compound after cleavage of its acetate group by intracellular esterases and this compound binds to ROS. As controls, AMB and FLZ treatments were used. The treatment was performed as described apoptosis/necrosis assay section. After the incubation period, the samples were washed, following suspension in 500 µL of PBS, and transferred to cytometer tubes. Then, 1.5 µL of the H2DCFDA solution was added with subsequent incubation at 25°C in the dark for 10 minutes. The samples were analyzed on a BD FACS Canto I flow cytometer.

Data Analysis
All data from this study are representative of at least three independent and triplicate experiments. GraphPad Prism 5.0 software (GraphPad Software Inc., La Jolla, CA) was used to construct graphs and for statistical analysis. Non-linear semi-log regression was performed to obtain the IC50 for treated HaCat cells. Analysis of variance with Bonferroni post-hoc test was applied to the other graphs. Differences with p < 0.05 were considered statistically significant.

RESULTS
Determination of MIC and MFC
The results of the antifungal activities of 2-chalcone, TRB, and FLZ against dermatophyte species are shown in Table 1. In all
strains tested, 2-chalcone had a MIC of 7.8 mg/L. TRB had a MIC of 0.03 mg/L in T. rubrum strains and 0.008 mg/L in T. mentagrophytes strain. On the contrary, T. rubrum ATCC 28189 was resistant to FLZ with a MIC of 64 mg/L, whereas the remaining strains, T. rubrum ATCC MYA-4438 and T. mentagrophytes ATCC 11481, were susceptible to FLZ (with MIC 4 mg/L and 1 mg/L, respectively).

The MFC for 2-chalcone corresponded to 15.6 mg/L, double the MIC, in all strains tested. The same trend was observed in the TRB results. FLZ had a MIC of 16, 32, and > 64 mg/L in T. mentagrophytes ATCC 11481, T. rubrum ATCC MYA-4438, and T. rubrum ATCC 28189, respectively.

**Effect of 2-Chalcone and Antifungal Drugs on Early-Stage and Mature Biofilms**

From 15.6 mg/L, 2-chalcone inhibited the metabolic activity of early-stage biofilms of the three strains tested, with approximately 90% reduction in cell viability when compared to the control without treatment (p < 0.001) (Figure 1A). TRB inhibited early-stage biofilms of T. mentagrophytes ATCC 11481, T. rubrum ATCC MYA-4438, and T. rubrum ATCC 28289 from concentrations of 0.06, 1, and 32 mg/L, respectively (p < 0.001) (Figure 1B). However, FLZ only inhibited the biofilms formed by the T. mentagrophytes ATCC 11481 from 32 mg/L (p < 0.001) (Figure 1C).

In mature biofilms, 2-chalcone showed potent anti-biofilm activity, and inhibited the metabolic activity by about 90% in all fungi tested from the concentration of 31.25 mg/L (p < 0.001) (Figure 2A). In contrast, TRB and FLZ did not show anti-biofilm activity at all the different concentrations tested (Figures 2B, C).

**Effect of 2-Chalcone and Antifungal Drugs on Planktonic Cells at a Concentration of 10^6 cells/mL**

The effect of 2-chalcone and of the drugs TRB and FLZ on planktonic cells was verified at the same concentration used for biofilm formation (1 x 10^6 cells/mL) by measuring the metabolic activity of the cells. The results showed that 2-chalcone could reduce the metabolic activity of T. rubrum ATCC 28189 from the concentration of 7.8 mg/L (p < 0.001) of T. rubrum ATCC MYA-4438 and T. mentagrophytes ATCC 1148 from 15.6 mg/L (p < 0.001) (Figure 3A). TRB was potent in T. mentagrophytes ATCC 1148 with a reduction in metabolic activity from the concentration of 0.008 mg/L, and in T. rubrum ATCC MYA-4438 and T. rubrum ATCC 28189 from 0.03 mg/L and 0.06 mg/L, respectively (p < 0.001) (Figure 3B). Besides, FLZ was less potent than TRB, with reduced metabolic activity from the concentration of 64, 256, and 512 mg/L in T. mentagrophytes ATCC 1148, T. rubrum ATCC MYA-4438, and T. rubrum ATCC 28189, respectively (p < 0.001) (Figure 3C).

### Table 1 | Antifungal activity (expressed in mg/L) of 2-chalcone, terbinafine, and fluconazole against dermatophyte species.

|                      | T. rubrum ATCC 28189 | T. rubrum ATCC MYA-4438 | T. mentagrophytes ATCC 11481 |
|----------------------|----------------------|------------------------|-----------------------------|
|                      | MIC      | MFC       | MIC     | MFC       | MIC    | MFC       |
| 2-Chalcone           | 7.8      | 15.6      | 7.8     | 15.6      | 7.8    | 15.6      |
| TRB                  | 0.03     | 0.06      | 0.03    | 0.06      | 0.008  | 0.016     |
| FCZ                  | >64      | >64       | 4       | 32        | 1      | 16        |

TRB, terbinafine; FCZ, fluconazole; MIC, minimum inhibitory concentration; MFC, minimum fungicidal concentration.

**Effect of Photodynamic Therapy on Planktonic Cells and on Early-Stage and Mature Biofilms**

The photodynamic therapy assay was applied using 2-chalcone as a photosensitizer against planktonic cells (10^6 cells/mL) and against early-stage and mature biofilms of the strains T. rubrum ATCC 28189, T. mentagrophytes ATCC 11481, and T. rubrum ATCC MYA-4438. The use of 2-chalcone as a photosensitizer for PDT was found to be effective against dermatophytes (Figure 4). The metabolic activities of planktonic cells of all tested species were inhibited from the concentration of 2 mg/L (Figure 4A), corresponding to a four-times reduction in concentration compared to its effect in the dark. The same reduction was observed in early-stage biofilms with inhibition from 4 mg/L (Figure 4B) and in mature biofilms with inhibition from 7.8 mg/L (Figure 4C).

**Scanning Electron Microscopy of Biofilms Treated With 2-Chalcone in the Dark and Mediated PDT**

SEM was used to evaluate the damage to in vitro early-stage and mature biofilms treated with 2-chalcone. For this analysis, the concentrations of 2-chalcone (in the dark and irradiated) determined in the susceptibility test were used. The topographies of biofilms treated with 2-chalcone confirmed the findings of the XTT reduction assay and showed a total collapse in the hyphal cell wall probably due to leakage of cytoplasmic content. These damages were observed in both early-stage biofilms (Figure 5A) and mature biofilms (Figure 5B). Further, evident inhibition of biofilm maturation and decreased presence of polysaccharide material was observed in the initial biofilms. On the contrary, untreated biofilms showed a dense network of interconnected hyphae embedded in an extracellular matrix (Figures 5A (e) B (a, c, e)).

Photomicrographs of T. rubrum ATCC 28189 biofilms treated with 2-chalcone-mediated PDT (Figure 6) also confirmed the XTT assay findings. However, the biofilms were less dense, and the collapse of the hyphae cell walls was less prominent (Figures 6C, D). Biofilms treated at a dose of 150 J/cm^2 without the photosensitizer (Figures 6A, B) showed similar results to those without treatment in the dark, also corroborating the XTT assay results.
Cytotoxicity Assay With 2-Chalcone in HaCat

Treatment of keratinocytes with 2-chalcone in the dark, reduced viability by almost 50% at 125 mg/L compared to the control without treatment (Figure 7). Cells treated with 2-chalconemediated PDT showed high toxicity with only 3% viability at the same concentration. After calculating the SI, 2-chalconemediated PDT presented better values in planktonic cells...
FIGURE 4 | Effect of 2-chalcone-mediated PDT using LED irradiation at a dose of 150 J/cm² in planktonic cells (10⁶ cell/mL) (A), early-stage (B), and mature biofilms (C) of T. rubrum ATCC 28189, T. mentagrophytes ATCC 11481, and T. rubrum ATCC MYA-4438, measured by the XTT reduction assay. Potentiation of 2-chalcone was shown when it was mediated PDT resulting in the inhibition of planktonic forms and biofilms (early-stage and mature) from 2, 4, and 7.8 mg/L. (\(p < 0.05; \**p < 0.01; \***p < 0.001\)).
FIGURE 5 | Scanning electron microscopy (SEM) images of early stage (A) and mature biofilms (B) of *T. rubrum* ATCC 28189, *T. rubrum* ATCC MYA-4438, and *T. mentagrophytes* ATCC 11481 untreated (a, c, e) and treated with 2-chalcone in the dark (b, d, f). The images of untreated biofilms show a robust biofilm, formed with the entanglement of integral hyphae and covered with a polymeric extracellular matrix (red arrows). Biofilms treated in the early-stage with 2-chalcone present a low density showing the action of 2-chalcone in inhibiting their maturation. In mature biofilms, as in the early-stage biofilms, 2-chalcone promoted total hyphal collapse (blue arrows).

FIGURE 6 | Scanning electron microscopy images of mature *T. rubrum* ATCC 28189 biofilms treated with 2-chalcone combined with blue LED at a dose of 150 J/cm² (C, D) and irradiated only at a dose of 150 J/cm² without the photosensitizer (A, B). The blue arrows indicate empty spaces within the biofilm showing that the biofilm had become less dense. The red arrows show collapsed hyphae.
than in the mature biofilm (9.92) when compared to the 2-chalcone treatment in the dark (Table 2). Photoexcitation of the compound using a light source is promising as photosensitization resulted in significant potentiation and decreased cell toxicity.

**Determination of the Mechanisms of Action**

**Verification of Damage to the Cell Membrane and Wall**

The results of ergosterol quantification in the fungal membrane (Figure 8A) showed that 2-chalcone in the dark, as well as 2-chalcone-mediated PDT, reduced the amount of total sterols extracted compared to the control (p < 0.01). These findings revealed that ergosterol inhibition might be a mechanism of action for 2-chalcone. FLZ and AMB also reduced the number of sterols extracted from the membrane (p < 0.001).

Confocal microscopy images (Figure 8B) of cells treated with 2-chalcone showed discontinuous staining of the cell wall and structural compromise compared to the control indicating that 2-chalcone can damage cell wall chitin and/or cellulose. Cells treated with FLZ showed no change in the cell walls of the conidia and hyphae.

**Quantification of ROS and Apoptosis/Necrosis**

In the dark, 2-chalcone induced ROS generation when compared to the control (p < 0.001) (Figure 9); however, 2-chalcone-mediated PDT did not produce ROS. AMB and H$_2$O$_2$ also induced ROS formation when compared to the untreated control (p < 0.001). In contrast, FLZ did not induce ROS formation (Figure 9).

Cells treated with 2-chalcone in the dark presented high necrosis levels (53–56.5%) compared to death by apoptosis (18.4–32.3%) (p < 0.001) (Figure 10A). When 2-chalcone was excited by a light source, almost all cell death was found to be due to necrosis (Figure 10B). AMB caused death through both mechanisms, apoptosis (p < 0.05) and necrosis (p < 0.001). In contrast, most FLZ-treated cells remained alive because the *T. rubrum* ATCC 28189 strain was resistant to FLZ as shown in the susceptibility assay. However, a tendency of cellular death mainly by necrosis, was observed (Figure 10).

**DISCUSSION**

Extensive research has been conducted on new anti-dermatophyte drugs (Gupta; Foley; Versteeg, 2017b; Gnat;
Lagowski; Nowakiewicz, 2020; Iwanaga et al., 2020) as microorganisms are increasingly developing resistance to conventional drugs with the hypothetical formation of biofilms in onychomycosis and high rates of recurrence and reinfection (Gupta; Daigle; Carviel, 2016; Gupta; Foley, 2019). Further, the increasing human infections by zoophilic species, mainly in tinea capitis and tinea unguium, are generally more challenging to treat and require systemic treatment (Gnat; Lagowski; Nowakiewicz, 2020).

The present work showed the anti-dermatophyte and anti-biofilm action of a compound derived from chalcone, a molecule of natural origin that is abundant in fruits and vegetables and has relatively simple laboratory synthesis. The compound shows enhanced action mediated PDT, with a better SI in human keratinocytes in the context of its effect in the dark. Tests of susceptibility and MFC determination showed the potent action of 2-chalcone with a MIC of 7.8 mg/L in all tested strains. Although the action of chalcones varies widely with their structural replacement pattern, natural and synthetic chalcones have already shown antifungal activity against Candida spp (Tavares et al., 2011), Cryptococcus gattii (Palanco et al., 2017), Paracoccidioides brasiliensis (Medina-Alarcón et al., 2020), H. capsulatum (Melo et al., 2017), and against dermatophytes (López et al., 2001; Gupta; Jain, 2015), proving to be potential candidates as future antifungal drugs. The MFC is defined as the lowest concentration of the drug necessary to inhibit 99.9% of fungal growth (Gil-Alonso et al., 2019). Hazen (1998) considered an antifungal agent as fungicidal if the MIC and MFC relationship is not greater than four times. In all tested strains, 2-chalcone presented a potent fungicidal profile, with the relation between MIC and MFC equal to 2 times. The TRB and FLZ susceptibility of all strains corroborated with previous studies conducted by Costa-Orlandi et al. (2020).
Further, 2-chalcone also showed potent action against early-stage and mature biofilms, as well as the planktonic form with $10^6$ cells/mL with variations of only two dilutions. Costa-Orlandi et al. (2020) and Singulani et al. (2018) considered insignificant variations in dilutions up to two dilutions. These results are encouraging as no effect was observed with the drugs TRB and FLZ, mainly when used to treat mature biofilms. Some studies conducted by our group have already demonstrated the anti-biofilm activity of chalcones against fungal species such as *C. gattii* (Palanco et al., 2017) and *H. capsulatum* (Melo et al., 2017). Studies have been carried out to assess the anti-biofilm action of conventional drugs for treating dermatophytoises such as TRB,
FLZ (Costa-Orlandi et al., 2020), itraconazole, voriconazole, griseofulvin (Brihlante et al., 2018), econazole (Toukabri et al., 2018), and formulations of piroxicam-based shampoos (Santos; Dias-Souza, 2017). However, in most cases, 50 times the MIC is required to observe any biofilm inhibition (Brihlante et al., 2018). Planktonic forms (10^6 cells/mL) are more sensitive to drugs when compared to biofilms, confirming that these communities show increased resistance to conventional antifungals. The strain T. rubrum ATCC 28189 demonstrated resistance to FLZ even in the planktonic form as described previously (Costa-Orlandi et al., 2020).

PDT is a relatively affordable treatment when used as an inexpensive photosensitizer and can be implemented in hospitals without incurring high costs (Bagla et al., 2015). Some studies have shown the fungicidal effect of photodynamic therapy against T. rubrum and T. mentagrophytes using CO_2 (De Oliveira et al., 2015) and toluidine (Baltazar et al., 2013), as well as with hypericin, hypocrellin, and curcumin as photosensitizers against Candida spp. (Davies et al., 2016; Yang et al., 2019). Cyclic chalcones have significant characteristics for effects in PDT (Melo et al., 2017). However, the photosensitizing properties of chalcones can be easily lost by the modification of their structures. For instance, Zhuang et al. (2018) reported that the introduction of a methyl group at the α position of the unsaturated ketone resulted in the loss of fluorescence. The usage of 2-chalcone as a photosensitizer in PDT showed that the compound did not lose its photosensitizing properties. Further, its effect was enhanced compared to that in the dark with a reduction in the MIC to a fourth part against planktonic cells, early-stage and mature biofilms.

Photosensitization with 2-chalcone showed increased toxicity in HaCat cells, with an IC_{50} reduction by almost by half. Similar results have been reported by Melo et al. (2017) when irradiated chalcone derivatives were co-incubated with NOX, HepG2, and HaCat cell lines at a dose of 12 and 42 J.cm^{-2}. The researchers also found that the dose of light was directly proportional to the increase in cell toxicity. In contrast, the SI in the treatment of 2-chalcone mediated PDT was higher than that in the dark treatment. The SI is the ratio of the IC_{50} and MIC (Scorzoni et al., 2016). This index indicates a compound’s selectivity between a fungal and host cell, to evaluate the relationship between safety and potency (Bagla et al., 2014; Scorzoni et al., 2016). SI values higher than ten are considered more specific (Ochoa-Pacheco et al., 2017; Singulani et al., 2019). Treatment of 2-chalcone-mediated PDT proved to be safer, with SI values ranging from 9.92 to 38.72 in mature biofilms and planktonic forms, respectively. These results are a consequence of the decreased MIC values in planktonic cells and biofilms, and the reduced toxicity in HaCat cells.

The antifungal properties of chalcones depend on their structural replacement pattern, as well as on the fungal genotype and cell density (López et al., 2001; Illicachi et al., 2017). The action on the biosynthesis of β- (1,3) glucan and chitin of the fungal cell wall (Gupta; Jain, 2015; Illicachi et al., 2017) has already been demonstrated, also the inhibition of the glutathione-S-transferase (GST) family, that are enzymes involved in drug resistance (Illicachi et al., 2017). Due to the diversity of mechanisms underlying the action of chalcones, our work verified the integrity of structures such as cell walls and cell membranes as well as the functional imbalance including oxidative stress induction and cell death mechanism in the treated samples. Treatment with 2-chalcone in the dark and 2-chalcone-mediated PDT reduced membrane ergosterol contents. The action of chalcones on this molecule has not been reported so far. Lack of ergosterol alters membrane fluidity, causing an increase in permeability and consequent osmotic imbalance (Ouf et al., 2013). Cells treated with AMB and FLZ also showed lower amounts of ergosterol because these drugs act by binding directly to membrane ergosterol and inhibiting its synthesis, respectively (Singulani et al., 2019). Further, our results showed deformation of the cell wall structure when stained with calcofluor white, a non-specific fluorophore that binds cellulose and chitin mainly in beta 1-3 and beta 1-4 polysaccharides, and emit fluorescence when excited (Harrington; Hageage, 2003). The action of chalcones on the fungal cell wall has been reported previously (Gupta; Jain, 2015; Illicachi et al., 2017). Reduced ergosterol and impaired cell wall structure confirm the findings in the SEM images showing that the hyphae were fully collapsed with a “pressed cell” appearance.

Treatment with 2-chalcone in the dark induced significant ROS generation. Drugs such as amphotericin B have ROS induction as a secondary mechanism of action, in addition to the main mechanism via ergosterol (Singulani et al., 2019). ROS generation depends on the drug’s ability to reach the intracellular region (Yoo; Ha, 2012). Possibly, a part of the compound that reached the cytoplasm was responsible for ROS generation. However, a significant part acts on the cell wall and membrane. Treatment with AMB as well as H_2O_2 induced ROS production. Further, AMB caused death by apoptosis as well as necrosis. Similar results were reported by Singulani et al. (2019) in C. neoformans. The presence of oxygen in the PDT takes the photosensitizer to an excited state with high reactivity, facilitating interactions with the surrounding molecules (Kwiathowski et al., 2018; Donohoe et al., 2019). These interactions can be type I or type II. Type I reactions result in free radicals whereas type II interactions induce formation of ROS such as singlet oxygen (1O_2), superoxide, hydrogen peroxide, and hydroxyl radical (Shibu et al., 2013; Zhang et al., 2018a). Our results showed that 2-chalcone mediated PDT promoted low ROS induction. Considering these facts, we speculate that the photosensitized 2-chalcone probably induces free radicals, through type I reactions.

Apoptosis and necrosis are the main mechanisms of cell death in cytotoxic responses to PDT. These depend on the photosensitizer nature, light dose, and cell type (Yoo; Ha, 2012). Treatment with 2-chalcone-mediated PDT almost entirely induced fungal death by necrosis. Necrosis is usually associated with a high concentration of photosensitizer and/or light dose, severe cell damage, and photosensitizers with tropism to the cell membrane (Yoo; Ha, 2012). A relatively high dose (150 J.cm^{-2}) was used in our assays, and the results showed that 2-chalcone has a tropism for the fungal membrane and cell wall. Induction of apoptotic death by the compound in the dark is probably a consequence of ROS generation. However, most cells
died from necrosis, which may be a result of other cell targets such as the cell wall and the fungal membrane ergosterol.

CONCLUSION

Our results showed that 2-chalcone is a molecule with anti-dermatophyte and anti-biofilm properties. When mediated PDT, its effect is enhanced, causing low toxicity to human skin keratinocytes and high SI value. Further, the compound targets specific fungal structures and promotes ROS generation, resulting in cell death from apoptosis and necrosis. Overall, this study contributes significantly to the discovery of new compounds with anti-biofilm activity, and other studies are being conducted to prove these findings both ex vivo and in vivo.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article-supplementary material. Further inquiries can be directed to the corresponding author.

REFERENCES

Aggarwal, N., and Goindi, S. (2012). Preparation and Evaluation of Antifungal Efficacy of Griseofulvin Loaded Deformable Membrane Vesicles in Optimized Guinea Pig Model of Microsporum Canis - Dermatophytosis. Int. J. Pharm. 437 (1–2), 277–287. doi: 10.1016/j.ijpharm.2012.08.015

Aggarwal, R., Targhotra, M., Kumar, B., Sahoo, P. K., and Chauhan, M. K. (2020). Treatment and Management Strategies of Onychomycosis. J. Mycol. Med. 30 (100949), 1–15. doi: 10.1016/j.jmscmed.2020.100949

Anéke, C., Otranto, D., and Cafaíchia, C. (2018). Therapy and Antifungal Susceptibility Profile of Microsporum Canis. J. Fungi 4 (107), 1–14. doi: 10.3390/jof4030107

Arthington-skaggs, B. A., Jradi, H., Desai, T., and Morrison, C. J. (1999). Quantitation of Ergosterol Content: Novel Method for Determination of Flucloxacilin Susceptibility of Candida Albicans. J. Clin. Diag. Res. 37 (10), 3332–3337. doi: 10.1128/JCM.37.10.3332-3337

Baccilar, I. O. L., Tshbone, T. M., Pavan, C., and Baptista, M. S. (2015). Photodynamic Efficiency: From Molecular Photochemistry to Cell Death. Int. J. Mol. Sci. 16, 20523–20559. doi: 10.3390/ijms160920523

Bagla, V. P., Mcgaw, L. J., Elgorashi, E. E., and Elof, J. N. (2014). Antimicrobial Activity, Toxicity and Selectivity Index of Two Biflavonoids and a Flavone Isolated From Podocarpus Henkelii (Podocarpaceae ) Leaves. Bio Med. Cent Complement Altern. Med. 14 (388), 2–7. doi: 10.1186/1472-6882-14-383

Baltazar, L. M., Soares, B. M., Carneiro, H. C. S., Avila, T. V., Ferreira, L., and Gouveia, L. F., et al. (2013). Photodynamic Inhibition of Trichophyton Rubrum: in Vitro Activity and the Role of Oxidative and Nitrosative Bursts in Fungal Death. J. Antimicrob. Chemother. 68, 354–361. doi: 10.1093/jac/dks414

Brillant, R. S. N., Correia, E. E. M., Guedes GM de, M., de Oliveira, J. S., Castelo-Branco D de, S. C. M., Cordeiro R de, A., et al. (2018). In Vitro Activity of Azole Derivatives and Griseofulvin Against planktonic and Biofilm Growth of Clinical Isolates of Dermatophytes. Mycoses. 61 (7), 449–454. doi: 10.1111/ myc.12763

Burkhalter, C. N., Burkhart, C. G., and Gupta, A. K. (2002). Dermatophytoma: Recalibration to Treatment Because of Existence of Fungal Biofilm. J. Am. Acad. Dermatol. 47 (4), 629–631. doi: 10.1067/mjd.2002.124699

Cai, C. Y., Rao, L., Rao, T., Guo, J. X., Xiao, Z. Z., Cao, J. Y., et al. (2017). Analoge of Xanthones-Chalcones and Bis-Chalcones as Alpha-Glucosidase Inhibitors and Anti-Diabetes Candidates. Eur. J. Med. Chem. 130, 51–59. doi: 10.1016/j.ejmech.2017.02.007

AUTHOR CONTRIBUTIONS

NB, CC-O, CF, and MM-G conceived and designed the study. NB, CC-O, CV, and JB performed all the experiments. NB, CV, and CC-O analyzed the data and wrote the manuscript. LA and LR synthesized 2-chalcone. All authors contributed to the article and approved the submitted version.

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Trichophyton Spp. Mycopathologia. 178 (3-4), 221–225. doi: 10.1007/s11046-014-9797-6

Pierce, C. G., van Apuluri, P., Tristan, A. R., Wormley, F. L. J., Mowat, E., Ramage, G., et al. (2008). Lopez-Ribot JL. A Simple and Reproducible 96-Well Plate-Based Method for the Formation of Fungal Biofilms and Its Application to Antifungal Susceptibility Testing. Nat. Protoc. 3 (9), 1494–1500. doi: 10.1038/nprot.2008.141

Santos, R. M., and Dias-Souza, M. V. (2017). Effectiveness of Five Antidandruff Cosmetic Formulations Against Planktonic Cells and Biofilms of Dermatophytes. Saudi J. Biol. Sci. 24 (2), 331–337. doi: 10.1016/j.sjbs.2015.09.033

Scorzoni, L., Benaducci, T., Almeida, A. M. F., Silva, D. H. S., Bolzani, V. D. S., and Gianinetti, M. J. M. S. M. (2007). The Use of Standard Methodology for Determination of Antifungal Activity of Natural Products Against Medical Yeasts Candida Sp and Cryptococcus Sp. Braz. J. Microbiol. 38 (3), 391–397. doi: 10.1590/S1517-83822007000300001

Scorzoni, L., Sangalli-Leite, F., de Lacorte Singulani, J., de Paula e Silva, A. C. A., Costa-Orlandi, C. B., Fusco-Almeida, A. M., et al. (2016). Searching New Antifungals: The Use of in Vitro and in Vivo Methods for Evaluation of Natural Compounds. J. Microbiol. Methods 123, 68–78. doi: 10.1016/j.mimet.2016.02.005

Shah, M. S., Najam-ul-Haq, M., Shah, H. S., Farooq Rizvi, S. U., and Iqbal, J. (2018). Quinoline Containing Chalcone Derivatives as Cholinesterase Inhibitors and Their in Silico Modeling Studies. Comput. Biol. Chem. 76, 310–317. doi: 10.1016/j.compbiolchem.2018.08.003

Shamali, N., Preußl, A., Sultsman, I., Mahammed, A., Gross, Z., Däschlein, G., et al. (2018). In Vitro Photodynamic Inactivation (PDI) of Pathogenic Germs Inducing Onychomycosis. Photodiagnostics Photodyn. Ther. 24, 358–365. doi: 10.1016/j.pdpt.2018.11.002

Shibu, E. S., Hamada, M., Murase, N., and Biju, V. (2013). Nanomaterials Formulations for Photothermal and Photodynamic Therapy of Cancer. J. Photochem Photobiol C Photocem Rev. 15 (1), 53–72. doi: 10.1016/j.jphotchemrev.2012.09.004

Singh, G., Arora, A., Kalra, P., Maurya, I. K., Ruizc, C. E., Estebanc, M. A., et al. (2018B). A Strategic Approach to the Synthesis of Ferrocene Appended Chalcone Linked Triazole Allied Organosulphonates: Antibacterial, Antifungal, Antiparasitic and Antioxidant Studies. Bioorg. Med. Chem. 27 (1), 188–195. doi: 10.1016/j.bmc.2018.11.038

Singh, A., Masih, A., Khurana, A., Singh, P. K., Gupta, M., Hagen, F., et al. (2018a). High Terbinfine Resistance in Trichophyton Interdigitale Isolates in Delhi, India Harbouiring Mutations in the Squalene Epoxydase Gene. Mycoses. 61 (7), 477–484. doi: 10.1111/myc.12772

Singulani J. de L., Galenele, M. C., Ramos, M. D., Gomes, P. C., dos Santos, C. T., de Souza, B. M., et al. (2019). Antifungal Activity, Toxicity, and Membranolytic Action of a Mastoparan Analog Peptide. Front. Cell Infect. Microbiol. 9, 419. doi: 10.3389/fimmu.2019.00419

Singulani, J. L., Scorzoni, L., Lourenccetti, N. M. S., Oliveira, L. R., Concolaro, R. S., da Silva, P. B., et al. (2018). Potential of the Association of Dodecyl Gallate With Nanostructured Lipid System as a Treatment for Paracoccidioidomycosis: in Vitro and in Vivo Efficacy and Toxicity. Int. J. Pharm. 547 (1-2), 630–636. doi: 10.1016/j.ijpharm.2018.06.013

Sun, K., Song, X., Jia, R. Y., Yin, Z., Zou, Y., Li, L., et al. (2017). In Vivo Evaluation of Galla Chinensis Solution in the Topical Treatment of Dermatophytosis. Evidence-Based Complement Evid Based Complement Alternat Med. 2017, 3843595. doi: 10.1155/2017/3843595

Tajudddeen, N., Isah, M. B., Sule, M. A., van Heerden, F. R., and Ibrahim, M. A. (2018). the Chemotherapeutic Potential of Chalcoalges Against Leishmanias: A Review. Int. J. Antimicrob. Agents 51 (3), 311–318. doi: 10.1016/j.ijantimicag.2017.06.010

Tavares, L. D. C., Johann, S., Maria De Almeida Alves, T., Guerra, J. C., Maria De Souza-Fagundes, E., Cisalpino, P. S., et al. (2011). Quinolinyl and Quinolinol N-Oxide Chalcoals: Synthesis, Antifungal and Cyctotoxic Activities. Eur. J. Med. Chem. 46 (9), 4448–4456. doi: 10.1016/j.ejmech.2011.07.019

Toukabri, N., Corpologno, S., Bougnoux, M. E., El Euch, D., Sadfi-Zaououi, N., and Simonetti, G. (2018). in Vitro Biofilms and Antifungal Susceptibility of Dermatophyte and Non-Dermatophyte Moulds Involved in Foot Mycosis. Mycoses. 61 (2), 79–87. doi: 10.1111/mmc.12706

Yang, Y., Hou, W., Liu, S., Sun, K., Li, M., and Wu, C. (2018). Biodegradable Polymer Nanoparticles for Photodynamic Therapy by Biodiimence Resonance Energy Transfer. Biomacromolecules. 19 (1), 201–208. doi: 10.1021/acs.biomac.7b01469

Yang, Y., Wang, C., Zhuge, Y., Zhang, J., Xu, K., Zhang, Q., et al. (2019). Photodynamic Antifungal Activity of Hypocrella Against Candida Albicans. Front. Microbiol. 10, 1810. doi: 10.3389/fmicb.2019.01810

Yoo, J. O., and Ha, K. S. (2012). New Insights Into the Mechanisms for Photodynamic Therapy-Induced Cancer Cell Death. Int. Rev. Cell Mol. Biol. 295, 139–174. doi: 10.1016/B978-0-12-394306-4.00010-1

Yuan, Y., Liu, Z., Jin, H., Sun, S., Liu, T., Wang, X., et al. (2017). Photodynamic Antimicrobial Chemotherapy With the Novel Amino Acid-Porphyrin Conjugate 4I : in Vitro and in Vivo Studies. PLoS One 12 (5), e0176529. doi: 10.1371/journal.pone.0176529

Zhang, J., Jiang, C., Longo, J. P. F., Azevedo, R. B., Zhang, H., and Muelmann, L. A. (2018a). an Updated Overview on the Development of New Photosensitizers for Anticancer Photodynamic Therapy. Acta. Pharm. Sin. B. 8 (2), 137–146. doi: 10.1016/j.apsb.2017.09.003

Zhang, X., Rakesh, K. P., Bukhari, S. N. A., Balakrishna, M., Manukumar, H. M., and Qin, H. L. (2018b). Multi-Targetable Chalcone Analogs to Treat Deadly Alzheimer’s Disease: Current View and Upcoming Advice. Bioorg. Chem. 80, 86–93. doi: 10.1016/j.bioorg.2018.06.009

Zhan, P., and Liu, W. (2017). The Changing Face of Dermatophytic Infections Worldwide. Mycopathologia. 182 (1-2), 77–86. doi: 10.1007/s11046-016-0882-8

Zhange, C., Zhang, W., Sheng, C., Zhang, W., Xing, C., and Miao, Z. (2018). Chalcone: A Privileged Structure in Medicinal Chemistry. Chem. Rev. 117 (12), 7762–7810. doi: 10.1021/acs.chemrev.7b00002

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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