Design, synthesis, and evaluation of novel 2-substituted 1,4-benzenediol library as antimicrobial agents against clinically relevant pathogens

Daiane Flores Dalla Lana a, Bruna Gerardon Batista a, Gabriella da Rosa Machado a, Mário Lettieri Teixeira b, Luís Flávio Souza de Oliveira c, Michel Mansur Machado c, Saulo Fernandes de Andrade d, William Lopes e, Marilene Henning Vainstein e, Alejandro Peixoto de Abreu Lima f, Enrique Pandolfi f, Edilma Elayne Silva g, Alexandre Meneghello Fuentefria a, Gustavo Pozza Silveira g,*

a Laboratory of Applied Mycology, Department of Analysis, Faculty of Pharmacy, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil
b Laboratory of Biochemistry and Toxicology, Instituto Federal de Santa Catarina, Concórdia, SC, Brazil
c Center for Studies in Biochemistry, Immunology and Toxicology, Universidade Federal do Pampa, Uruguaiana, RS, Brazil
d Department of Raw Materials Production, Faculty of Pharmacy, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil
e Department of Molecular Biology and Biotechnology, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil
f Laboratorio de Síntesis Orgánica, Departamento de Química Orgánica, Facultad de Química, CP11800, Universidad de la República, Montevideo, Uruguay
g Department of Organic Chemistry, Institute of Chemistry, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil

A R T I C L E   I N F O

Article history:
Received 2 April 2019
Accepted 22 September 2019
Available online 25 September 2019

Keywords:
Fungi
ESKAPE pathogens
1,4-benzenediol derivatives
Antimicrobial activity
Mechanism of action
Toxicity

A B S T R A C T

Development of new antimicrobial agents, capable of combating resistant and multidrug-resistant fungal and bacterial clinical strains, is necessary. This study presents the synthesis and antimicrobial screening of 42 2-substituted-1,4-benzenediols, being 10 novel compounds. In total, 23 compounds showed activity against fungi and/or bacteria. Benzenediol compounds 2, 5, 6, 8, 11, and 12 demonstrated broad spectrum antimicrobial actions, including resistant and multidrug-resistant species of dermatophytes (Trichophyton mentagrophytes), Candida spp. and the ESKAPE panel of bacteria. Minimum inhibitory concentrations of these compounds for fungi and bacterial strains ranged from 25 to 50 μg/ml and 8–128 μg/ml, respectively. The antifungal mechanism of action is related to the fungal cell wall of dermatophytes and membrane disruption to dermatophytes and yeasts, in the presence of compound 8. Specific structural changes, such as widespread thinning along the hyphae and yeast lysis, were observed by scanning electron microscopy. The effects of compound 8 on cell viability are dose-dependent; however they did not cause genotoxicity and mutagenicity in human leukocyte cells nor haemolysis. Moreover, the compounds were identified as nonirritant by the ex-vivo Hen’s egg test-chorioallantoic membrane (HET-CAM). The furan-1,4-benzenediol compound 5 showed in vivo efficacy to combat S. aureus infection using embryo-nated chicken eggs. Therefore, the compounds 8, and 5 are promising as hits for the development of new antimicrobial drugs with reduced toxicity.

© 2019 The Authors. Published by Elsevier B.V. on behalf of King Saud University. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

1. Introduction

Fungal infections occur on the skin and mucous membranes causing invasive and systemic diseases in humans (Kim, 2016; Fuentefria et al., 2018). Dermatomycoses, or cutaneous mycoses, are the most prevalent fungal infections worldwide. Although these diseases are rarely life-threatening, they reduce the quality of life and are the main reason for patients to see a dermatologist (Pfaller et al., 2006; Nenoff et al., 2014; Dalla Lana et al., 2016). Between 2001 and 2011 in France, a retrospective study surveying human skin and nail infections showed that dermatophytes such as M. canis, M. gypseum, T. mentagrophytes, and T. rubrum were responsible for approximately 70% of cases of superficial infections
González-Bello et al., 2019). Another genus of filamentous fungi, Fusarium, another genus of filamentous fungi, is an emerging opportunistic fungal pathogen that causes local or systemic infections. Reports of multidrug-resistance are frequent. Hence, the successful use of a therapeutic drug, or combination of antifungal therapies against these species, is compromised (Batista et al., 2017).

*C. albicans* is the most frequently isolated fungus in humans (Li et al., 2013). However, an increase in infections caused by other Candida spp. has been observed, such as: *C. krusei*, *C. tropicalis*, *C. glabrata*, *C. guilliermondii*, and *C. parapsilosis* (Kaur et al., 2016). Fungal diseases caused by Candida spp. are not routinely diagnosed. Consequently, hospitalized patients, especially in intensive care units, are often inappropriately treated with broad-spectrum antibiotics. Bloodstream infection and invasive candidiasis are substantially more common than realized and probably result from multiple factors, including: unrestrained antibiotic use, indwelling devices, immunocompromised patients, and renal support (Denning et al., 2017).

The Infectious Diseases Society of America classified a panel of six pathogens capable of escaping the biocidal action of drugs and responsible for a large part of the nosocomial infections around the world as ESKAPE. This acronym is used as reference to the group of bacteria: *E. faecium*, *S. aureus*, *K. pneumonia*, *A. baumannii*, *P. aeruginosa*, and *Enterobacter* spp. (Pendleton et al., 2013). Most of these strains are multidrug-resistant which makes the development of new drugs to combat infections caused by this panel of pathogens imperative (Santajit and Indrawattana, 2016; González-Bello et al., 2019).

Polyphenols are commonly found in nature and demonstrate a remarkable range of biological activities (Harborne et al., 1999; Whitting, 2001; Dai and Mumper, 2010; Oksana et al., 2012; De Arias et al., 2012; Soto-Hernandez et al., 2017). The potent in vitro and in vivo pharmacological activity of the 2,5,4-trihydroxy ybenzylglyz against *Leishmania* spp. was reported (Roldos et al., 2008; Serna et al., 2015). This result motivated us to synthesize a library of 2-substituted-1,4-benzenediols and investigate their antimicrobial effect. Therefore, this study presents the antimicrobial activity of new 2-substituted-1,4-benzenediol derivatives against a panel of clinical fungal and bacteria strains, as part of our ongoing program for the identification of new leads for the development of new antimicrobials (Silveira et al., 2012; Batista et al., 2017). In addition, the target and mechanism of action of the 1,4-benzenediol on fungal cells and the toxicity of these hits were investigated. Finally, an alternative in vivo *S. aureus* (ATCC 25923) model of infection followed by the treatment using a 1,4-benzenediol was demonstrated.

## 2. Materials and methods

### 2.1. Chemicals

In total, 42 1,4-benzenediol derivatives were evaluated for antifungal and antibacterial activity. The synthesis of compounds 1–4, 9–18, 22, 23, 25–31, 36, and 39 (Lima et al., 2016), 5, 19, 32, 33, and 38 (Rolón et al., 2019), 20, and 40 (Ozaki et al., 1997a, 1997b) were previously demonstrated (Ozaki et al., 1997a, 1997b; Lima et al., 2016; Rolón et al., 2019). General procedures for preparation of novel compounds 6–8, 21, 24, 34, 35, 37, 41, and 42, and the hydrogen characterization data of other compounds that not yet been presented (20 and 40) are shown in the supp info. All compounds were dissolved in dimethyl sulfoxide (DMSO) and diluted using sterile ultrapure water to give solutions with DMSO concentrations lower than 1% for subsequent investigation of biological activities, mechanism of action and toxicity.

### 2.2. In vitro susceptibility tests

#### 2.2.1. Antifungal agents

Terbinafine (TBF), amphotericin B (AMB), and anidulafungin (AFG), purity $\geq 97\%$, were supplied by Cristialia (Sao Paulo, Brazil), griseofulvin (GSF), purity $\geq 97\%$, was acquired from Wallace Pharmaceutical (Mumbai, India), ketoconazole (KTC), purity $\geq 96\%$, was obtained from All Chemistry (Sao Paulo, Brazil), fluconazole (FLC) purity $\geq 98\%$ was obtained from Sigma-Aldrich (Sao Paulo, Brazil), itraconazole (ITC) purity $\geq 97\%$, was supplied by Casarà laboratory (Dist. PHARMOS; Buenos Aires, Argentina), miconazole (MCZ) purity $\geq 97\%$, was supplied by Valdequimica Chemical Products (Sao Paulo, Brazil), and voriconazole (VRC); purity $\geq 98\%$, was supplied by Pfizer (Sao Paulo, Brazil). The preparation of stock and work solutions followed the recommendations of the CLSI (M38-A2, 2008). Commercial antifungals were used as references.

#### 2.2.2. Fungal strains

Candida spp. (C. albicans - CA ATCC 18804, C. krusei - CK 02*, C. glabrata - CG 09*, and C. tropicalis - CT 72A*), dermatophytic filamentous fungi (M. canis - MCA 01, M. gypseum - MGY 42, F. mentagrophytes - TME 16*, and T. rubrum - TRU 45), and other filamentous fungi (C. fujikuroi – F2 and F. solani – F9) were selected to evaluate the antifungal activity of the 1,4-benzenediols. Some clinical strains are resistant or multidrug-resistant (*). For Candida spp. the resistance was defined based on Kuriyama et al. (2005) and the Clinical and Laboratory Standards Institute (CLSI) breakpoints (M27-A3, 2008; M27-S4, 2012) considering, in this case, the minimum inhibitory concentration (MIC) values: CK 02* resistant to ITC (MIC = 1 µg/ml) and FLC (MIC = 64 µg/ml), and ketoconazole (MIC = 4 µg/ml) as well as MCZ (MIC = 8 µg/ml) resistant, and CT 72A* resistant to ITC (MIC = 1 µg/ml), MCZ (MIC > 8 µg/ml), and VRC (MIC = 2 µg/ml). As for dermatophytes, the resistance (in the sense of reduced susceptibility) was established considering the following resistance threshold concentrations (MICs): terbinafine ($\geq 1.0\mu g/ml$), griseofulvin ($\geq 4.0\mu g/ml$), and ketoconazole ($\geq 8.0\mu g/ml$). Clinical isolate TME 16* was considered multidrug-resistant (MIC terbinafine = 4 µg/ml, MIC griseofulvin > 32 µg/ml and MIC ketoconazole = 16 µg/ml). *C. fujikuroi* and *F. solani* are resistant to itraconazole and fluconazole (MICs > 128 µg/ml). All the clinical and reference strains used in this study are deposited in the Mycology Collection of the Federal University of Rio Grande do Sul, Porto Alegre, RS, Brazil.

Susceptibility tests were performed for determining the MIC and the minimum fungicidal concentration (MFC). The MIC was determined by the broth microdilution method, according to the protocol established for yeasts - M27-A3 (CLSI, 2008) and filamentous fungi - M38-A2 (CLSI, 2008). The inoculum of yeasts (0.5x10^3 to 2.5x10^1 CFU/ml) and filamentous fungi (1.0x10^3 to 3.0x10^2 CFU/ml) were prepared from cultures grown on sabouraud dextrose agar (SDA; Kasvi, Curitiba, Brazil) and potato dextrose agar (PDA; Neogen, Lansing, USA), respectively. The assays were conducted in duplicate, with RPMI medium, containing L-glutamine (without sodium bicarbonate; Gibco, USA), buffered to pH 7.0 with 0.165 M of 3-(N-morpholino)propanesulfonic acid (MOPS; Neon, São Paulo, Brazil), with subsequent incubation at 35 °C. The analysed concentrations of the synthetic compounds ranged from 0.03 to 50 µg/ml. The reading of the results for *Candida* spp. and *Fusarium* spp. was performed in 24–48 h, and for dermatophytes after 96 h of incubation. MIC was defined as the lowest concentration of the substance capable of inhibiting the visible fungal growth. Sterility control (drug-free medium only) and positive control (inoculum and culture medium only, for evaluation of the fungal cell viability) were used.

To determine the MFC, aliquots of each serial microdilution (corresponding to MIC, 2xMIC and 4xMIC) were spread on
SDA (Candida) and PDA (filamentous fungi), which were incubated at 35 °C and analysed. The MFC was defined as the lowest drug concentration that yielded up to three colonies (i.e., >99% of the inoculum was killed) (Espinell-Ingroff et al., 2002). The assay was performed in triplicate.

2.2.3. Antibacterial agents
Oxacillin, ampicillin, gentamicin and imipenem purity ≥97% were purchased from Sigma-Aldrich (Sao Paulo, Brazil).

2.2.4. Bacterial strains
E. coli (ATCC 25922), E. faecalis (ATCC 29212) and (ATCC 51299), as well as ESKAPE panel of pathogens including strains of S. aureus (ATCC 33591) and methicillin-susceptible as well as ESKAPE panel of pathogens including strains of S. aureus (ATCC 25923), K. pneumoniae (ATCC700603), P. aeruginosa (ATCC 27853), A. baumannii (ATCC 19606), A. baumannii (10C 3174), and E. aerogenes (ATCC 13048) were obtained by donation from Instituto Oswaldo Cruz, RJ, Brazil. Evaluation of MICs followed the CLSI microdilution method using BBL™ Mueller Hinton II broth (Interlab, Brazil) as described previously - M100 (CLSI, 2018). Briefly, two-fold serial dilutions of each compound were prepared in triplicate in 96-well plates and inoculated with 5 × 10^5 CFU/ml of the bacterial suspension. Plates were incubated at 37 °C for 16–20 h (CLSI, 2018). Antibiotics abovementioned were used as controls.

2.3. In vivo antifungal efficacy in Infected Egg Test-Chorioallantoic Membrane (IET-CAM)

Fresh and fertile white eggs (Lohmann selected Leghorn, LSL) were kept under optimized incubation conditions (38–39 °C, 55–60% humidity, 12 days). The preparation of S. aureus strain (ATCC 25923) inoculum followed the M100 document recommendations (CLSI, 2018). On the 4th day, the CAM of eggs were infected with 0.1 ml inoculum 4×/day and the infectious process confirmed by the visualization of colonies onto the CAM (holes sealed with parafilm). The treatment started on the 8th day of incubation. An aliquot of 0.1 ml of the compound 5 (64 μg/ml, 0.5% DMSO solution) or 0.1 ml 0.9% NaCl (negative control) was added onto the CAM. On the 12th day, eggs were opened and 0.1 ml aliquot removed and spread into Blood agar. Then, the plates were incubated at 35 °C for 48 h, for subsequent counting of CFU/ml (Jacobsen et al., 2010). This study is approved by the Animal Ethics Committee (CEUA), protocol number 4/2016 (Instituto Federal Catarinense, Concórdia, Brazil). The experiment was carried out in triplicate.

2.4. Investigation of antifungal mechanism of action

2.4.1. Sorbitol assay
The effect of the benzenediol derivatives on the integrity of the fungal cell wall was evaluated by sorbitol protection assay (Frost et al., 1995, Escalante et al., 2008). MICs were determined by the standard broth microdilution following the documents M27-A3 and M38-A2 (CLSI, 2008) in the absence and presence of different concentrations (50–200 mg/ml) of external ergosterol (Sigma-Aldrich, St. Louis, MO, USA) added to the RPMI 1640 growth medium. The plates were incubated at were incubated at 35 °C for 168 h. AMB was used as positive control. MICs were measured after 2 and 5 days for Candida spp.; and after 4 and 7 days for dermatophytes (Escalante et al., 2008; Carrasco et al., 2012). Experiments were carried out in triplicate.

2.4.2. Ergosterol assay
The ability of the benzenediol derivatives to complex with ergosterol in the fungal membrane was evaluated by ergosterol binding assay. The MICs were determined by the standard broth microdilution following the documents M27-A3 and M38-A2 (CLSI, 2008) in the presence of different concentrations (50–200 mg/ml) of external ergosterol (Sigma-Aldrich, St. Louis, MO, USA) added to the RPMI 1640 growth medium. The plates were incubated at were incubated at 35 °C for 168 h. AMB was used as positive control. MICs were measured after 2 and 5 days for Candida spp.; and after 4 and 7 days for dermatophytes (Escalante et al., 2008; Carrasco et al., 2012). Experiments were carried out in triplicate.

2.4.3. SEM analysis
The experiment followed the M27-A3 and M38-A2 guidelines (CLSI, 2008): C. albicans and M. canis adhered cells obtained from the susceptibility test were washed with phosphate buffer saline (PBS). Then, 500 μl glutaraldehyde (2.5%, type 1, Sigma Aldrich) was poured into the cells and the mixture diluted with 50 ml sodium cacodylate (0.1 M, pH 7.2, Sigma Aldrich) and kept for 1 h at room temperature. Next, the cells were washed three times with sodium cacodylate (0.1 M, pH 7.2, 10 ml) containing sucrose (0.2 M) and MgCl2 (2 mM). Adhered cells were dehydrated using solutions of ethanol (30, 50 and 70%, for 5 min, then 95% and 100% (2×) for 10 min). Samples were subjected to critical point drying (EM CPD 300, Leica) immediately after dehydration, mounted on metallic stubs, sputter-coated with a 15–20 nm gold-palladium layer, and visualized in a scanning electron microscope (Carl Zeiss EVO® MA10, Oberkochen, Germany) operating at 10 kV. Concentration evaluated 8 (25 μg/ml). Drug controls: TBF (0.016 μg/ml for M. canis) and ITc (1 μg/ml for C. albicans).

2.4.4. Epifluorescence microscopy
C. albicans and M. canis samples (approximately 200 μl) treated with the compound 24 (50 μg/ml) from the susceptibility test were used to epifluorescence microscopy analysis (Silveira et al., 2017; Sood et al., 2017). This mixture was placed over a slide and analysed by the epifluorescent microscope (Bel Photonic®) (8-green filter, C-UV filter) equipped with a CCD blacklight camera. Photos were taken with a digital camera (Nikon model D600, macro mode, 60 mm, f/3.2D) after 60 s (dermatophytes) and 5 s (Candida spp.) (Barros et al., 2016).

2.5. Toxicity evaluation

2.5.1. Cell culture, cytotoxicity, genotoxicity and mutagenicity
Cell cultures of human leukocytes were prepared using venous blood collected by venepuncture from a male volunteer (number of the approval protocol of the Ethics Committee: 23.081.005770/009-38). Aliquots (1 ml) of whole blood were immediately transferred to 10 ml of RPMI 1640 medium supplemented with 1% phytohaemagglutinin, 10% foetal bovine serum and 1% streptomycin/penicillin (Montagner et al., 2010). Subsequently, cell culture treatments were performed with compound 8 (25 μg/ml and 50 μg/ml, in 0.5% DMSO), hydrogen peroxide solution (H2O2, 100 μM, positive control), and PBS (negative control). Cell cultures were incubated (CO2 incubator for cell culture, 5% CO2, Model MCO-19AIC, Sanyo) at 37 °C for 72 h (Montagner et al., 2010). Cytotoxic, genotoxic, and mutagenic parameters were established in triplicate. Cell viability was assessed with 0.2% trypan blue (Sigma-Aldrich), according Burrow et al. (1998). Genotoxicity was performed by the comet assay. Cells were classified from 0 (no visible damage) to 4 (maximum damage) to provide the damage index (DI) from 0 to 400 (Singh et al., 1988). The assessment of mutagenicity was by Panótico Rápido” (Laborclin). In total, 100
cells were counted per slide. All particles within the cells were separated from the nucleus and counted as micronuclei (MN) (Thomas et al., 2008; Montagner et al., 2010).

2.5.2. Haemolysis assay
Fresh rabbit blood was mixed with the anticoagulant K2-EDTA. Rabbit erythrocytes were harvested by centrifugation for 5 min at 400 rpm and washed three times with PBS buffer. Serial dilutions of 1,4-benzenediol derivatives 2, 5, 8, and 12 (25–100 μg/ml) were prepared and incubated with 1% erythrocyte suspension (PBS) for 15 min at 37 °C. Then, the cells were spun down by centrifugation and the supernatant transferred to a 96-well plate. Next, the absorbance (650 nm) was measured using a microplate spectrophotometer (BioTek Instruments). PBS (negative, 0% haemolysis) and water (positive, 100% haemolysis) were used as controls.

2.5.3. Hen’s egg test-chorioallantoic membrane (HET-CAM)
Fresh and fertile white eggs (Lohmann selected Leghorn, LSL) were kept under optimized incubation conditions (38–39 °C, 55–60% humidity, 10 days). On the 10th day, the eggshell, around the airspace, was removed with a rotary tool (Dremel, WI). Subsequently, 0.3 ml of the compounds 2, 5, and 12 (64 μg/ml, 0.5% DMSO solution), 8 (50 μg/ml, 0.5% DMSO solution) and controls (negative control: 0.9% NaCl; positive control: 0.1 M NaOH) were added to the CAM of the eggs (Pammi et al., 2013). The irritant effect was observed at three times: 30 sec, 2 min, and 5 min after application of substances and controls. The result of the irritation score (IS) was calculated according to the Eq. (1) (Pammi et al., 2013) and presents a maximum value of 21. The eggs were analysed in relation to the appearance of haemorrhaging, lysis and protein coagulation, respectively. The assay was performed in triplicate.

\[
\text{IS} = \left( \frac{(301 - \text{hemorrhage time})}{300} \right) \times 5 \\
+ \left( \frac{(301 - \text{lysis time})}{300} \right) \times 7 \\
+ \left( \frac{(301 - \text{coagulation time})}{300} \right) \times 9 \\
\text{Eq. (1). Formula for the determination of irritation score (IS)}
\]

2.6. Statistical analysis
Differences between controls and treatments were statistically analysed by ANOVA followed by the Tukey’s test (p < 0.05 was considered statistically significant). Data analysis was performed using the GraphPad Prism 5.0 software.

3. Results

3.1. Chemicals

3.1.1. Synthesis of 2-substituted 1,4-benzenediols
We developed a synthetic method for the synthesis of 2-substituted 1,4-benzenediols (Lima et al., 2016; Rolón et al., 2019) based on green chemistry principles (Anastas and Warner, 1998). This strategy involves a tandem aldolic condensation/isomerization/aromatization between 1,4-cyclohexanediol and the selected aldehyde, to give 2-substituted-1,4-benzenediol derivatives with moderate to excellent yields (see supp. info; Scheme S1 and S2). Unfortunately, the nitro group could not be introduced because of synthetic unsuitability. Our groups are focusing in preparing the CF₃ derivative to support SAR observations and the results will be reported in the due course (see supp. info.). All spectral and chemical data for the new molecules are available at the supp. info. (Figs. S1–S30).

3.1.2. General procedure for the synthesis of (E)-2-(2,5-dihydroxy styryl) quinoline:
Diacetoxystyryl quinolines were prepared based on Polanski’s method (Polanski et al., 2002). Methylquinoline derivatives were treated with 2,5-dihydroxybenzaldehyde in acetic anhydride to obtain the desired diacetoxystyryl analogues after an aldolic condensation reaction. This procedure takes advantage of using the acidity of the methyl group of methylquinolines. Then, the diacetoxy derivatives obtained were hydrolysed in sulfuric acid (12 M) under reflux until the reaction was completed by thin layer chromatography (TLC) analysis (see supp. info.; Scheme S4).

3.1.3. General procedure for the synthesis of 2-(2,5-dihydroxyphenethyl) quinoline derivatives:
2,5-Dihydroxyphenethyl quinoline derivatives were synthesized from the respective unsaturated starting material under usual catalytic hydrogenation conditions (see supp. info.; Scheme S5). Surprisingly, low yields were obtained, which might be associated with the quinolone moiety absorption over the charcoal used as a support for the metal catalyst during catalysis.

3.2. In vitro susceptibility tests

3.2.1. Fungal and bacterial strains
Eight strains were used to determine the MICs and the MFCs of 42 1,4-benzenediol derivatives. In total, 12 compounds showed antifungal activity: 1–12 (Table 1). 1,4-Benzenediols 1 and 10 were fungicide for all Candida spp. analysed at 50 μg/ml. Derivative 7 was fungistatic at 25 μg/ml for the whole panel of yeasts, which was the lowest MIC observed for the library. Compounds 2, 4, and 6 exhibited action only against dermatophytes, being fungistatic at 50 μg/ml. Meanwhile, 1,4-benzenediols 9 and 11 proved to be fungicides at 50 μg/ml. Table 1. The furan derivative 5 was active against just one Candida strain (CA ATCC 18804) and the whole panel of dermatophytes tested. Compounds 3 and 12 inhibited the growth of G. fujikuroi and F. solani (50 μg/ml). These two strains are resistant to ITC and FLC (MICs > 128 μg/ml). Finally, the fluoroquinoline derivative 8 (fungicide, MICs = MFCs of 50 μg/ml) demonstrated broad spectrum antifungal activity against the entire panel of dermatophytes and yeasts tested. This panel includes the multidrug-resistant and resistant species of T. mentagrophytes, C. krusei, C. glabrata, and C. tropicalis (MICs and breakpoints of commercial antifungal agents are presented in Table 1).

Next, 17 compounds were found to be active (MICs ≤ 128 μg/ml) against the Gram-positive bacteria (S. aureus methicillin sensitive (ATCC 25923), S. aureus methicillin resistant (ATCC 33591), E. faecalis (ATCC 29212), and E. faecium (ATCC 51299)) from the ESKAPE panel of pathogens: 2, 5, 6, 8, 11–23. The furan derivative 5 demonstrated the highest activity of the library of 1,4-benzodiolis (MICs 8–64 μg/ml, Table 2). 1,4-Benzenediols 2, 5, 19, and 20 were also active (MICs ≤ 64 μg/ml) against E. coli (ATCC 25922), which is used as a model for identification of molecules that could inhibit the growth of Gram-negative bacteria, Table 2.

1,4-Benzenediols 2, 5, 19, and 20 were screened against the entire Gram-negative ESKAPE panel. The 4-chlorophenyl derivative 2 presented moderate activity (32–128 μg/ml) against the strains tested. This substance showed a broad spectrum of action inhibiting the growth of Gram-negative and Gram-positive bacteria from...
the ESKAPE panel. Meanwhile, the furan benzenediol 5 demonstrated an MIC of 8 μg/ml to K. pneumoniae (ATCC 700603) being the most active molecule of the library on this strain, Table 3 (see Figs. 1 and 2).

Another 19 1,4-benzenediols synthesized neither showed antifungal activity at 50 μg/ml for Gibberella fujikuroi (F2) and Fusarium solani (F9), which are resistant to ITC and FLC (MICs > 128 μg/ml).

**3.3. Antifungal mechanism of action**

**3.3.1. Sorbitol assay**

The influence of sorbitol on the MICs of the vinyl-fluoroquinoline line-1,4-benzenediol 8 against the whole panel of dermatophytes and Candida spp. were evaluated, using the AFG as antifungal control. For dermatophytes, after 168 h of incubation, MICs (Table 1) to 8 folded once in the presence of sorbitol, whereas for AFG the MICs (Table 1) increased more than four times (Table S1). MICs changed more abruptly when sorbitol was added to the yeast medium in the presence of compound (Table S1). MICs increased up to 4–8-fold after adding a total of 1,4-benzenediols 3 and 12 showed antifungal activity at 50 μg/ml for Gibberella fujikuroi (F2) and Fusarium solani (F9), which are resistant to ITC and FLC (MICs > 128 μg/ml).

**Table 1**

| Compound | Dermatophytes | Candida spp. |
|----------|--------------|--------------|
|          | MCA 01 | MGY 42 | TME 16 | TRU 45 | CA ATCC 18804 | CK 02 | CG 09 | CT 72A |
| 1        | >50>50 | >50>50 | >50>50 | >50>50 | 50>50 | 50>50 | 50>50 | 50>50 |
| 2        | 50>50 | 50>50 | 50>50 | 50>50 | >50>50 | >50>50 | >50>50 | >50>50 |
| 3        | 50>50 | 50>50 | 50>50 | 50>50 | >50>50 | >50>50 | >50>50 | >50>50 |
| 4        | 50>50 | 50>50 | 50>50 | 50>50 | >50>50 | >50>50 | >50>50 | >50>50 |
| 5        | 50>50 | 50>50 | 50>50 | 50>50 | >50>50 | >50>50 | >50>50 | >50>50 |
| 6        | >50>50 | >50>50 | >50>50 | >50>50 | 25>50 | 25>50 | 25>50 | 25>50 |
| 7        | 50>50 | 50>50 | 50>50 | 50>50 | 50>50 | 50>50 | 50>50 | 50>50 |
| 8        | >50>50 | >50>50 | >50>50 | >50>50 | >50>50 | >50>50 | >50>50 | >50>50 |
| 9        | 50>50 | 50>50 | 50>50 | 50>50 | >50>50 | >50>50 | >50>50 | >50>50 |
| 10       | >50>50 | >50>50 | >50>50 | >50>50 | >50>50 | >50>50 | >50>50 | >50>50 |
| 11       | 50>50 | 50>50 | 50>50 | 50>50 | >50>50 | >50>50 | >50>50 | >50>50 |
| 12       | >50>50 | >50>50 | >50>50 | >50>50 | >50>50 | >50>50 | >50>50 | >50>50 |
| TBF      | 0.03 (S) | 0.03 (S) | 0.03 (S) | 0.03 (S) | 0.03 (S) | 0.03 (S) | 0.03 (S) | 0.03 (S) |
| GSF      | 1 (S) | 1 (S) | 1 (S) | 1 (S) | 1 (S) | 1 (S) | 1 (S) | 1 (S) |
| KTC      | 0.5 (S) | 1 (S) | 1 (S) | 1 (S) | 1 (S) | 1 (S) | 1 (S) | 1 (S) |
| FLC      | – | – | – | – | – | – | – | – |
| ITC      | – | – | – | – | – | – | – | – |
| MCZ      | – | – | – | – | – | – | – | – |
| VRC      | – | – | – | – | – | – | – | – |

| Compound | Bacteria  |
|----------|-----------|
|          | S. aureus ATCC 25923* | S. aureus ATCC 33591 | E. faecalis ATCC 51299 | E. faecalis ATCC 29212 | E. coli ATCC 25922 |
| 13       | >128 | 8 | >128 | >128 | >128 |
| 14       | >128 | 32 | >128 | >128 | >128 |
| 15       | >128 | 8 | >128 | >128 | >128 |
| 16       | >128 | 64 | >128 | >128 | >128 |
| 17       | >128 | 8 | >128 | >128 | >128 |
| 18       | >128 | 64 | >128 | >128 | >128 |
| 19       | 128 | 64 | >128 | >128 | >128 |
| 20       | 128 | 64 | 16 | >128 | >128 |
| 5        | 8 | 8 | 64 | 64 | 64 |
| 6        | 64 | 16 | 128 | 128 | 128 |
| 8        | >128 | 64 | >128 | >128 | >128 |
| 21       | >128 | 64 | 32 | >128 | >128 |
| 11       | >128 | >128 | 8 | >128 | >128 |
| 22       | >128 | 32 | 128 | 128 | 128 |
| 12       | >128 | 8 | 16 | >128 | >128 |
| 23       | >128 | 32 | 16 | >128 | >128 |
| oxacillin | 0.5 | 1 | 1 | 1 | 1 |
| ampicillin | 1 | 1 | 1 | 1 | 1 |

* Resistant to methicillin.
Table 3

MICs (µg/ml) of 1,4-benzenediol derivatives against Gram-negative ESKAPE panel bacteria.

| Compound | K. pneumoniae ATCC700603 | P. aeruginosa ATCC 27853 | A. baumannii ATCC 19606 | A. baumannii IOC 3174 | E. aerogenes ATCC 13048 |
|----------|--------------------------|--------------------------|--------------------------|------------------------|------------------------|
| 2        | 64                       | 32                       | 64                       | 64                     | 128                    |
| 5        | 8                        | >128                      | >128                      | >128                   | >128                   |
| 19       | >128                     | >128                      | >128                      | >128                   | >128                   |
| 20       | 128                      | 128                       | 64                       | 128                    | 128                    |
| Antibiotic | >128<sup>a</sup>   | 1<sup>b</sup>             | 2<sup>c</sup>             | 2<sup>c</sup>          | 1<sup>c</sup>          |

IOC: Instituto Oswaldo Cruz.

<sup>a</sup> Ampicilin.

<sup>b</sup> Gentamicin.

<sup>c</sup> Imipinem.

Fig. 1. 1,4-Benzenediol derivatives (1–12) presented in Tables 1–3.

Fig. 2. 1,4-Benzenediol derivatives (13–23) presented in Tables 2 and 3.
expected, the AMB drug control demonstrated an ergosterol dependent effect. For instance, after adding 200 \( \mu \)g/ml of ergosterol, MIC of AMB against \( C. \) tropicalis jumped from 0.5 to \( >128 \) \( \mu \)g/ml. The same trend was observed for the dermatophytes. However, MICs increased more discretely in the case of filamentous fungi (twice for compound 8 and up to 16 times for AMB, after 7 days and maximum concentration of ergosterol). Data are present in the supp. info., Table S2.

### 3.3.3. SEM analysis

\( C. \) albicans was in vitro treated separately with ITC (drug control) and compound 8. It was noticed that before the addition of the substances, \( C. \) albicans cells presented the expected oval shape without alteration (Fig. 3A). After treatment with ITC, changes in cell shape and size were remarkable (Fig. 3B; arrows point to damaged cells). The same was observed in the cells in the presence of 8. This compound appeared to lyse yeast cells (Fig. 3C; arrows point to damaged cells). Intact hyphae of \( M. \) canis (dermatophyte) were observed, and are indicated in Fig. 3D. After treatment with TBF, hyphae collapsed (Fig. 3E; arrows point to damaged cells). The same was observed for the vinyl fluoroquinoline-benzenediol 8 (Fig. 3F; arrows point to damaged cells).

### 3.3.4. Epifluorescence microscopy

\( C. \) albicans and \( M. \) canis were treated with the fluorescent vinyl-fluoroquinoline-1,4-benzenediol 24 which was inactive against both fungi (MICs > 50 \( \mu \)g/ml, Table 1). After 5 min, fungi cells were observed under white light using an epifluorescence microscope equipped with a digital camera. The characteristic oval and filamentous shapes of yeasts and filamentous fungi cells were noted, respectively. Then, fungi cells were exposed to an ultraviolet light source with a 365 nm wavelength. Pictures were taken successively after 5 s for \( C. \) albicans and 60 s for \( M. \) canis using the confocal microscope (Fig. 4, B-D and G-I, respectively). The last photo was taken of \( M. \) canis after 300 s from the beginning of the experiment (Fig. 4). The quinoline derivative 24 was able to penetrate into both fungi cells (Fig. 4, B-D and G-I) and deteriorated much faster inside \( C. \) albicans than \( M. \) canis due to the UV-light (Fig. 4).

### 3.4. Toxicity evaluation

#### 3.4.1. Cytotoxicity, genotoxicity and mutagenicity

The viability of human leukocytes was approximately 70% in the presence of the vinyl-fluoroquinoline 8 (50 \( \mu \)g/ml), Fig. 5(a). Meanwhile, PBS used as negative control demonstrated approximately 100% cell viability, Fig. 5(a–c). However, at this concentration (50 \( \mu \)g/ml), compound 8 did not cause significant DNA damage (Fig. 5(c)) and was not mutagenic (since there was no significant micronucleus frequency after in vitro treatment with this compound (Fig. 5(b)).

#### 3.4.2. Haemolysis

The mean percentage of rabbit erythrocytes haemolysis (±standard deviation (SD)) of 1,4-benzenediol derivatives 2, 5, 8, and 12, at 100 \( \mu \)g/ml (concentration was two times higher than MIC) were 4.22 ± 0.0045, 4.25 ± 0.0052, 4.37 ± 0.0021 and 3.88 ± 0.0007, respectively.

#### 3.4.3. HET-CAM

The HET-CAM test resulted in an IS for the compounds 2 (64 \( \mu \)g/ml), 5 (64 \( \mu \)g/ml), 8 (50 \( \mu \)g/ml), and 12 (64 \( \mu \)g/ml) of 2.66, 2.48, 2.94, and 2.62, respectively (see supp. info. – Figs. S32 and S33), which classify these compounds as nonirritants, according to the methodology.

### 3.5. IET-CAM

The results of the IET-CAM showed eggs with an initial colony count of \( 10^2 \)–\( 10^3 \) CFU/ml for \( S. \) aureus (ATCC 25923). After the incubation period, the absence of bacterial growth in the plates after treatment with 64 \( \mu \)g/ml of the furan-benzenediol 5 (Fig. 6 B) was observed. In contrast, \( 10^4 \) to \( 10^5 \) CFU/ml for untreated eggs (Fig. 6 A) was noted.

### 4. Discussion

A library of 1,4-benzenediol derivatives were screened against eight fungi strains (four filamentous and four yeasts), as well as
ten Gram-positive and Gram-negative bacteria. This synthetic series was prepared by the reaction of an aldehyde with 1,4-cyclohexanediene, using green chemistry conditions: K$_2$CO$_3$ as a base, in water:ethanol (9:1) mixture as solvent. Twelve new compounds were prepared by this methodology (see supp. info.; Scheme S1).

The fluoroquinoline-1,4-benzenediol 8 was identified as the best line of attack to the fungi strains, with the fungicidal action at 50 μg/ml for the whole panel (Table 1). This compound was further evaluated to determine its mechanism of action using the sorbitol and ergosterol assays.

Sorbitol exerts osmotic protection to the fungi cell wall (Frost et al., 1995; Batista et al., 2017). Consequently, the addition of sorbitol to the medium will decrease antifungal activity, if the drug acts on this target. In the presence of sorbitol, MICs for 8 increased abruptly, indicating that this molecule acted on the fungal cell wall of the yeasts and filamentous fungi. This effect seems to be weaker on filamentous fungi since MICs were raised just two times their initial values.

Ergosterol is an essential chemical found on the fungal membrane. Drugs, such as AMB, have an affinity to sterols. Accordingly, it is possible to determine if a new antifungal candidate is able to act on the fungal cell membrane by supplying ergosterol to the culture medium (Pippi et al., 2015; Batista et al., 2017). Mammals count on cholesterol, instead of ergosterol, as an essential structural component of cells. Thus, targeting ergosterol is very attractive for developing selective antifungal agents (Pippi et al., 2015). The fluoroquinoline derivative 8 was shown to act on cell
membranes of dermatophyte and Candida spp., since MICs were increased in the presence of exogenous ergosterol.

SEM images verified specific structural changes for C. albicans and M. canis after in vitro treatment with compound 8 (25 μg/ml). For the dermatophyte, morphological modifications of tubular structures were noted with 8, when compared to the untreated control (Fig. 3F). For C. albicans, the cellular damage was observed on the blastocidium, which was fully lysed by 8 (Fig. 3C). The rupture of the cell could be related to the effect of the molecule on the cell wall and consequent death of the microorganism (fungalicidal effect). The same morphological damage in fungi cells was recently observed in related studies using SEM, after treating the strains with a variety of others organic compounds (Dalla Lana et al., 2015, 2018, 2019; Batista et al., 2017).

Epifluorescence microscopy was used as an auxiliary technique to observe the possible internalization of molecules into the fungal cell. Compound 8 presents two independent aromatic rings separated by two methylene carbons, which do not allow for electrons to flow over the whole aromatic system. Meanwhile, the presence of a double bond connecting the aromatic rings of compound 24 creates a strong UV–VIS absorption band not observed with 8. In addition, compound 24 presented neither a fungicidal nor fungistatic effect over Candida spp. or filamentous fungi employed in this study (MICs > 50 μg/ml, Table 1). Therefore, 24 was chosen as a fluorescent probe to test the hypothesis that 1,4-benzenediols are able to cross the cell wall and membrane of fungi cells. C. albicans and M. canis were treated with the fluorescent probe (compound 24) and observed by epifluorescence microscopy. A great number of cells of both fungi were noted with white light, as expected, since 24 is inactive (Fig. 4A and F). Then, after turning on the UV-light (365 nm), we noticed that cells lit up due to the presence of probe molecules inside them (Fig. 4B and C). The UV-light faded until pictures became fully dark (Fig. 4E and J). The UV-light degraded 24 much faster inside C. albicans, which indicates that M. canis cells demonstrated some level of UV protection.

Toxicological assays were performed using human leukocytes to identify levels of toxicity of compound 8. The fluoroquinoline-1,4-benzenediol 8 exhibited cell viability of 70% at 50 μg/ml, which is statistically similar to 100 μM (3.4 μg/ml) of hydrogen peroxide used as a positive control (Fig. 5(a–c)). However, the fluoroquinoline derivative 8 did not induce DNA damage, in the comet assay (Fig. 5(c)) which relates the genotoxicity potential of substances to affect the cell integrity and its genetic material (Batista et al., 2017). The micronucleus analysis also indicated that 8 is not capable of producing genetic mutations at 50 μg/ml (Fig. 5(b)). Additionally, the fluoroquinoline-1,4-benzenediol 8 did not cause haemolysis, even at 100 μg/ml (Section 2.4.2).

Toxicological studies using alternative models, such as HET-CAM it is also very advantageous to predict the toxicity of the compound to membranes (Batista et al., 2017). The HET-CAM is an ex vivo method alternative to the Draize rabbit eye test, which measures vascular changes in the CAM. The membrane is an analogue of the ocular conjunctive and responds to injuries caused by processes such as inflammation similarly to what is observed in the conjunctival tissue of rabbits eyes. Since substances are directly applied over the CAM, the results obtained are generally more practical than animal models (ICCVMA, 2010). Results from HET-CAM using embryonated eggs demonstrated that compounds 2, 5, 8, and 12 are nonirritants of the CAM (ICCVMA, 2010).

Electron donating groups present in the aryl rings of the benzenediols were shown to be deleterious for bacterial activities. On the other hand, the best results against S. aureus and E. faecalis (MICs < 32 μg/ml for at least two S. aureus and E. faecalis strains) were obtained for derivatives containing electron withdrawing groups such as halogens (compounds 18, Table 2, and 25–27, 31 and 36, supp. info.; Fig. S31 vs compounds 2, 13 and 17). A second aromatic ring, such as the one present on quinolines (compounds 6, 8 and 21, Table 2, and 24, 33, 34 and 42, supp. info.; Fig. S31) also resulted in benzenediol derivatives whose MICs ≥ 32 μg/ml for Gram-positive strains (Table 2). In fact, the presence of nitrogen practically did not increase Gram-positive activities (compounds 19, 20, 6, 8 and 21, Table 2 and 24, 29, 31, 34, 37, 38, 40, 41 and 42, see supp. info.; Fig. S31). The only exception was noted for the 4-aminoacetylphenyl-1,4-benzenediol 15 which demonstrated MICs 8–32 μg/ml for three Gram-positive strains (Table 2). Alkyl benzenediol derivatives were also active against Gram-positive bacteria (compounds 22, 12 and 23). Of these, the most promising was the hexyl-1,4-benzenediol 12 (MICs 8–64 μg/ml to all S. aureus and E. faecalis tested, Table 2). These results are similar to the ones obtained for the furan-1,4-benzenediol 5 (MICs 8–64 μg/ml, Table 2). Ma et al. (2019) investigated the antibacterial activity of hydroquinone and arbutin in Ainsliaea bonatii, which both displayed relatively strong antibacterial activity against S. aureus, MRSA, and extended spectrum β-lactamase S. aureus. Results showed that hydroquinone could destroy the bacterial cell wall and membrane, increase permeability, lead leakage of intracellular substance affect synthesis of protein, and influence expression of genes (Ma et al., 2019).

Although E. coli (ATCC 25922) is not included in the ESKAPE panel, due to its fast growth, this strain was chosen as a model for fast identification of molecules with Gram-negative potential. 1,4-Benzenediol derivatives 2, 5, 19, and 20, presented MIC ≤ 64 μg/ml against E. coli (Table 2). These compounds were further screened against the entire Gram-negative ESKAPE panel. The best activity was observed for the furan-1,4-benzenediol compound 5 against K. pneumoniae (ATCC 700603) (MIC 8 μg/ml, Table 3) which is known to be clinically resistant to most antibiotics. The thiophene-1,4-benzenediol 32 (5 isostere, supp. info.; Fig. S21) and the benzofuran-1,4-benzenediol 35 (5 with an extra ring, supp. info.; Fig. S31) were also prepared. However, both 1,4-benzenediols were inactive against all bacteria tested.

Since the furan-1,4-benzenediol 5 was identified as the best hit to Gram-positive strains and also active against K. pneumoniae (ATCC 700603), this molecule was chosen to have its potency tested in an in vivo experiment using the CAM. Thus, the CAM was infected with S. aureus (ATCC 25923) after four days of embryo development. Then, after another four days, a solution of compound 5 (64 μg/ml) was added over the CAM. On the 12th day of the experiment, eggs were opened to check the number of CFUs still present. The furan derivative 5 was able to kill the bacteria without affecting embryo development (Fig. 6B). Infected eggs not treated with the furan derivative 5 did not have normal embryo maturity (Fig. 6A). To our knowledge, this is the first time that this type of testing with chicken embryonated egg was used to assess the efficacy of a new synthetic compound to combat infection in vivo.

In vitro (Schlecht et al., 2015) and in vivo/ex vivo (Pammi et al., 2013) studies have demonstrated a synergistic effect as a consequence of co-infection of Candida spp. with some Gram-positive bacteria, such as Staphylococci or Enterococci. It was believed that Gram-positive bacteria adhere or bind to the hyphae of Candida spp. It was shown that C. albicans can transport S. aureus into tissues, disseminating the infection in an oral co-colonization model. Therefore, co-infection of Candida spp. and S. aureus results in a more serious infection than each microorganism alone (Pammi et al., 2013). Besides the Gram-positive activities of the furan benzenediol 5, this molecule was also active against dermatophytes screened (MICs ≤ 50 μg/ml, Table 1) and C. albicans (MIC 50 μg/ ml, Table 1). Therefore, as a study perspective, the furan derivative 5 could be used to prepare formulations to combat infections both associated with bacteria and fungi (Silveira et al., 2017). Besides,
S. aureus is one of the most virulent pathogens of skin and soft tissue infections. These infections may be local and progress to become invasive (Tong et al., 2015). Formulations containing the furan-1,4-benzenediol 5 could be applied for the treatment of chronic wound infections, where higher concentrations of compounds, such as 5, could be used to tackle Staphylococcus infections without cytotoxicity concerns. Finding new ways of treating wound infections is of major importance, since 1 to 2% of populations in developing countries will develop chronic skin wounds, which cost $ 25 billion/year just in the United States (Soo et al., 2017). The ability of antibiotics to cure bacterial infections is at a serious risk due to the emergence and worldwide spread of superbugs. A lack of innovation and investment for almost 50 years has led to significant efforts currently being devoted to find alternative and innovative therapies to face this challenge (González-Bello et al., 2019).

5. Conclusions
The present study determined the antifungal (filamentous fungi and yeasts of the genus Candida) and antibacterial activity (ESKAPE panel) of a novel library of 2-substituted-1,4-benzenediols easily synthesized in just one chemical step using inexpensive commercially available reagents and mild green chemistry conditions (K₂CO₃, ethanol and water). 1,4-Benzenediols demonstrated a broad spectrum of action and reduced toxicity to human leukocytes. The mechanism of antifungal action was identified targeting the cell wall and membrane of yeasts and dermatophytes. Benzenediol compounds 5 (for bacteria) and 8 (for fungi) were classified as the most promising for further in vivo studies. Although benzenediols presented moderate activities, 1,4-benzenediols 5, 12, and 22 showed an antimicrobial profile. In addition, the furan-1,4-benzenediol 5 was demonstrated to be active in an ex vivo model, as well as nonirritant to the mucous membranes. Therefore, the development of antimicrobials using 5 (or even 8 for fungicides) as the active component of antimicrobial formulations seems quite promising.

Declaration of Competing Interest
The authors declare that they have no conflict of interest.

Acknowledgments
The authors are grateful to the Center of Microscopy and Microanalysis-UFGRS for the SEM and the Brazilian agencies CAPES, CNPq, and FAPESGR (Edital PRONUNIQUE 2016) for the financial support. G.P.S. and E.P. are thankful for bilateral project CAPES-Udeler #049-2013. A. M. Fuenteferia is grateful to CNPq for the PQ fellowship. Finally, all authors are grateful to Anderson Ramos Carvalho for the graphic enhancement of the manuscript.

Authors’ contributions
Daiane F. Dalla Lana, Bruna Batista, Gabriella M. Machado, Mário L. Teixeira, Luis F.S. de Oliveira, Michel M. Machado, William Lopes, Marilene H. Vainstein Edilma E. Silva conceived, planned, and carried out the experiments. Saulo F. de Andrade, Alejandro Peixoto de Abreu Lima, Enrique Pandolfi, Gustavo P. Silveira contributed to sample preparation, and synthesis. Alexandre M. Fuenteferia and Gustavo P. Silveira contributed to the interpretation of the results and supervision of all the work. Daiane F. Dalla Lana took the lead in writing the manuscript. All authors provided critical feedbacks during the manuscript preparation.

Appendix A. Supplementary material
Supplemental data to this article can be found online at https://doi.org/10.1016/j.ijjpds.2019.09.003.

References
Anastas, P.T., Warner, J.C., 1998. Green Chemistry: Theory and Practice. University Press, Oxford.
Barlow, J.L., Marques, S.M.T., Stefani, V., 2016. The use of epifluorescence microscopy and fluorescent dyes for visualization of Oxyuris equi eggs. Vet. Parasitol. 226, 162–166.
Batista, B.G., Dalla Lana, D.F., Silveira, G.P., Sá, M.M., Ferreira, M., Russo, T.V.C., Canto, R.R.S., Barbosa, F.A.R., Braga, A.I., Kaminski, T.F.A., de Oliveira, L.F.S., Machado, M.M., Lopes, W., Vainstein, M.H., Teixeira, M.L., de Andrade, S.F., Fuenteferia, A. M., 2017. Allylic selenocyanates as new agents to combat Fusarium species involved with human infections. ChemistSelect. 2, 11926–11932.
Burrow, M.E., Weldon, C.B., Tang, Y., Nayar, G.L., Krajewski, S., Reed, J.C., Hammond, T.C., Clejan, S., Beckman, B.S., 1998. Differences in susceptibility to tumor necrosis factor alpha-induced apoptosis among MCF-7 breast cancer cell variants. Cancer Res. 58, 4940–4946.
Carrasco, H., Raimondi, M., Sveraz, L., Di Liberto, M., Rodrigue, M.V., Espinoza, L., Madrid, A., Zacchino, S., 2012. Antifungal activity of eugenol analogues. Influence of different substituents and studies on mechanism of action. Molecules 17, 1002–1024.
Chernet, R., Ferreira, L., Guilhot, J., 2008. Dermatophytoises in animals. Mycopathologia 166, 385–405.
CLSI, 2008. Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts. Approved Standard – Third Edition. CLSI document M27-A3. Clinical and Laboratory Standards Institute, Wayne, PA.
CLSI, 2008. Reference Method for Broth Dilution Antifungal Susceptibility Testing of Filamentous Fungi. Approved Standard – Second Edition. CLSI document M38-A2. Clinical and Laboratory Standards Institute, Wayne, PA.
CLSI, 2018. Performance standards for antimicrobial susceptibility testing, 25th ed. CLSI supplement M100, Clinical and Laboratory Standards Institute.
Dai, J., Mumper, R.J., 2010. Plant Phenolics: Extraction, analysis and their antioxidant and anticancer properties. Molecules 15, 7313–7352.
Dalla Lana, D.F., Donato, R.K., Bundchen, C., Guez, C.M., Bergamo, V.Z., de Oliveira, L.F.S., Machado, M.M., de Andrade, S.F., Mamm, H.S., Fuenteferia, A.M., 2015. Imidazol sulfox with antifungal potential against multidrug-resistant dermatophytes. J. Appl. Microbiol. 119, 377–388.
Dalla Lana, D.F., Batista, B.G., Alves, S.H., Fuenteferia, A.M., 2016. Dermatofitoses: agentes etiológicos, formas clínicas, terapêutica e novas perspectivas de tratamento. Clin, Biomed. Res. 36, 230–241.
Dalla Lana, D.F., Giuliani, L.M., Reolon, J.B., Lopes, W., Vainstein, M.H., Di Liberto, M., Bergamo, V.Z., de Oliveira, L.F.S., Machado, M.M., de Andrade, S.F., Sá, M.M., Ferreira, M., Munaretto, L.S., Cruz, L., Silveira, G.P., Elayne, E., Fuenteferia, A.M., 2018. Nanoemulsion improves the antifungal activity of allylic thiocyanates against yeasts and filamentous pathogenic fungi. ChemistSelect 3, 11663–11670.
Dalla Lana, D.F., Carvalho, Â.R., Lopes, W., Vainstein, M.H., Guimarães, L.S.P., Teixeira, M.L., de Oliveira, L.F.S., Machado, M.M., de Andrade, S.F., Sá, M.M., Russo, T.V.C., Silveira, G.P., Fuenteferia, A.M., 2018. Structure-based design of s-lactones for new antifungal drug development: susceptibility, mechanism of action, and toxicity. Folia Microbiol. 64, 509–519.
De Arias, A.R., Pandolfi, E., Veja, M.C., Rolón, M., 2012. Selected natural and synthetic phenolic compounds with antimelanoma activity: a five-year review. Curr. Bioact. Compd. 8, 307–333.
Denning, D.W., Perlin, D.S., Muldoon, E.G., Colombo, A., Chakrabarti, A., Richardson, M.D., Sorrell, T.C., 2017. Delivering on antifungal resistance agenda not possible without improving fungal diagnostic capabilities. Emerg. Infect. Dis. 23, 177–183.
Escalante, A., Gattuso, M., Perez, P., Zacchino, S., 2008. Evidence for the mechanism of action of the antifungal Phytolaccoside B Isolated from Phytolacca tenerrima Hauman. J. Nat. Prod. 71, 1720–1725.
Espinel-Ingroff, A., Forthgill, A., Peter, J., Rinaldi, M.G., Walsh, T.J., 2002. Testing fungal species of clinical significance in vitro and in vivo using a standardized broth microdilution method. J. Clin. Microbiol. 40, 3204–3208.
Espinel-Ingroff, A., Fothergill, A., Peter, J., Rinaldi, M.G., Walsh, T.J., 2002. Testing fungal species of clinical significance in vitro and in vivo using a standardized broth microdilution method. J. Clin. Microbiol. 40, 3204–3208.
Frost, D.J., Brandt, K.D., Cugier, D., Goldman, R., 1995. A whole-cell assembly. J. Antibiot. 48, 306–310.
Fuentefria, A.M., Pippi, B., Dalla Lana, D.F., Donato, R.K., de Oliveira, L.F.S., Machado, M.M., de Andrade, S.F., Sá, M.M., Ferreira, M., Munaretto, L.S., Cruz, L., Silveira, G.P., Elayne, E., Fuenteferia, A.M., 2018. Nanoemulsion improves the antifungal activity of allylic thiocyanates against yeasts and filamentous pathogenic fungi. ChemistSelect 3, 11663–11670.
González-Bello, C., 2019. Recently developed synthetic compounds with anti-infective activity. Curr. Opin. Pharmacol. https://doi.org/10.1016/j.coph.2019.01.004.
Harborne, J.B., Baxter, H., Moss, G.P., 1999. Phytochemical Dictionary: Handbook of Bioactive Compounds from Plants. Taylor & Francis, London.
Kaur, R., Dhakad, M.S., Goyal, R., Haque, A., Mukhopadhyay, G., 2016. Identification of...