Synergism and Antifungal Potential of the Flavonoid Diosmin Against Clinical Isolates of Candida spp.

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Article

Synergism and Antifungal Potential of the Flavonoid Diosmin Against Clinical Isolates of *Candida* spp.

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Abstract: Fungal infections caused by *Candida* spp. cause potentially fatal infections with high morbidity and mortality rates, particularly in immunocompromised individuals. Their resistance to conventional antifungal drugs is closely associated with biofilm formation, which increasingly limits available therapeutic options, making medical practice a major challenge worldwide. In this context, plant derived compounds are an excellent alternative to be investigated. The objective of this study was to evaluate the antifungal potential of the flavonoid diosmin (DIO) and its synergistic capacity with commercial antifungals against clinical isolates of *Candida* spp. Susceptibility testing by broth microdilution method showed antifungal activity of DIO against all isolates tested, with minimum inhibitory concentration (MIC_{90}) values ranging from 1150 to 2251 μg/mL. Furthermore, we report the ability of DIO to inhibit biofilm and mature biofilm formation, with a greater inhibitory effect on fungal biofilm formation; this effect with DIO was significantly greater in most cases than that shown by amphotericin B (AFB). Intracellular leakage experiments (260/280 nm) showed damage to the cell membrane. This indicates that the antifungal action of DIO could be associated with damage to the cell membrane integrity and consequent death of these pathogens. Synergism experiments with DIO and fluconazole (FLZ) and DIO and AFB revealed a significant synergistic effect against *Candida* spp. These results highlight the antifungal and synergistic potential of the flavonoid DIO against antifungal resistant *Candida* spp. Furthermore, these findings serve as a basis for future studies aimed at elucidating DIO's antimicrobial mechanisms of action and contribute to the search for novel compounds from natural sources with antimicrobial potential.

Keywords: flavonoid; diosmin; antifungal; *Candida* spp; antibiofilm; synergism

1. Introduction

Candida spp. are a common cause of healthcare associated infections (HAIs) globally; approximately 1565000 people contract invasive candidiasis each year, particularly immunosuppressed individuals, including: critically ill patients in intensive care units (ICUs), people on long term broad spectrum antibiotic therapy and, recently, people with complications of post-COVID-19 disease. Invasive candidiasis is often misdiagnosed and is responsible for 995000 deaths (63.6% of all cases) annually. Africa is estimated to have a higher proportion of the invasive candidiasis burden than the rest of the world [1-3]. Bloodstream infections due to these pathogens are associated with mortality of up to approximately 40% according to the *Excellence Center of Medical Mycology* (ECMM), Cologne, Germany [4,5]. Furthermore, *Candida* spp., are the most common cause of fungal endocarditis, implicated in more than 50% of cases, are associated with high morbidity and mortality (0.70%) and present numerous challenges during clinical care [6]. These pathogens can persist within the host through the development of pathogenicity and multidrug resistance, which often leads to the failure of therapeutic strategies. A particular feature of the pathogenicity of *Candida* spp. it is their ability to form biofilms, which protects them from external factors such as the host's immune system defenses and antifungal drugs, multiplying by more than 1000 their resistance to first-line antifungals, compared to the planktonic growth of the same strains [7,8]. The rapid spread

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of multi resistant *Candida* spp. strains [1,9-12], associated with therapeutic failure and high mortality is alarming, representing this situation as a substantial threat to the prognosis of patients. In this context, the search for and development of new compounds with activity against *Candida* spp. that are safe, tolerable, and effective is urgent today. Since ancient times, natural products, particularly plant-based ones, have made a valuable contribution to pharmacotherapy, especially in infectious diseases and cancer [13,14]. Plants play a primary role as a source of specialized metabolites with recognized medicinal properties, due to their wide chemical diversity, these metabolites can be used directly as bioactive compounds, as drug prototypes or used as pharmacological tools for different targets [15], which is why they are an excellent alternative to be investigated.

Flavonoids are phytochemicals widely known for their health promoting pharmacological properties, including antimicrobial activity [16]. Diosmin (DIO) (diosmetin 7-O-rutinoside), Figure 1, is a flavone glycoside derived from hesperidin, a flavanone found abundantly in citrus fruits; it has anti-inflammatory, antioxidant, antidiabetic, antihyperlipidemic, antifibrotic, anticancer and hepatoprotective effects, among others [17-21]. Regarding the antimicrobial activity of this flavonoid, its potential against *Pseudomonas aeruginosa* has been reported, reducing its virulence mechanisms [22]; *Escherichia coli, Pseudomonas putida, Staphylococcus aureus* [23], varicella-zoster virus (VZV) [24], and its synergistic effect with amoxicillin-clavulanic acid (AMC) against *Mycobacterium tuberculosis* [25], among other biological activities, however, its potential against pathogenic yeasts of the Candida genus has not been documented. We hypothesize that the flavonoid DIO has activity against *Candida* spp. and the fungal biofilms of these pathogens. The objective of this investigation was to evaluate the antifungal potential of DIO against clinical isolates of *Candida* spp., estimate their ability to inhibit biofilms and explore their possible effect on the yeast membrane, contributing to the search for new compounds of natural origin that can serve as adjuvants in the treatment of pathogenic yeasts resistant to antifungal drugs.

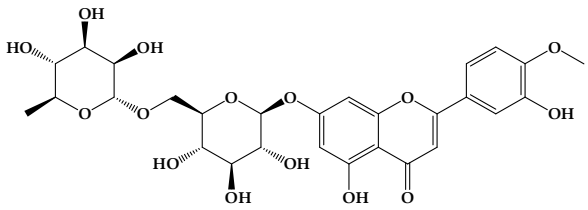

Figure 1. Structure of the flavonoid diosmin (DIO), diosmetin 7-O-rutinoside.

2. Results

2.1. Susceptibility Testing

DIO showed antifungal activity against all clinical isolates of *Candida* spp. studied. We observed a reduction in the growth percentage of yeasts treated with DIO, compared to untreated isolates used as controls. Figure 2 shows the similar trend among isolates to increase the percentage of growth reduction as the concentration of DIO increases. Table 1 shows the MIC values; MIC_{90} values of DIO were obtained between 1150 and 2251 μg/mL and MIC_{50} values between 660.4 and 1199 μg/mL. FLZ MIC_{90} values ranged from 2.3 to 241.8 μg/mL, with the majority of strains tested resistant to FLZ. This effect on *Candida* spp. was shown to be dependent on DIO concentration.

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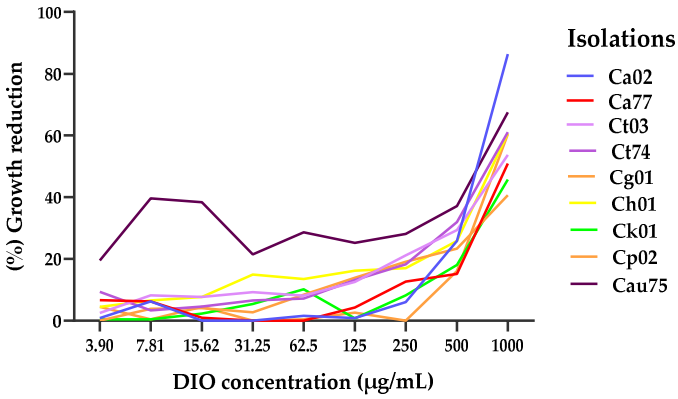

Figure 2. Growth reduction of *Candida* spp. isolates exposed to DIO (MIC_{90} of each isolate). A strong, positive linear relationship is observed between DIO concentration and the percentage reduction in *Candida* spp. growth; that is, as the DIO concentration increases, so does the percentage reduction in *Candida* spp. growth. This is consistent with the Pearson correlation coefficient ($0.82 < r < 0.99$) across all isolates. Furthermore, hypothesis testing for the correlation coefficient yielded a p-value < 0.05, indicating a significant linear relationship with 95% confidence.

Table 1. Minimum inhibitory concentration (MIC_{90}) values (µg/mL) of DIO vs FLZ against *Candida* spp.

Candida spp.	DIO MIC_{50}	DIO MIC_{90}	FLZ MIC_{90}
Ca02	660.4	1150	55.34
Ca77	1079	1952	2.3
Ct03	916	1756	154.5
Ct74	808.8	1526	241.8
Cg01	1199	2251	214.3
Ch01	853.2	1655	133.9
Ck01	1168	2118	69.99
Cp02	947	1670	42.09
Cau75	669.7	1786	75.65

It is observed that the efficacy of DIO was different between strains of the same species.

2.2. Biofilm Reduction

All *Candida* spp. isolates evaluated in this study produced biofilms on polystyrene microplates, as shown in Figure 3a; showing strong biofilm biomass production, the isolates *C. tropicalis*, *C. haemulonii* and *C. parapsilosis* ($OD_{590} > 3$), while the isolates *C. auris*, *C. albicans* and *C. krusei* (OD_{590}: 1.1-3.0) were moderate biofilm producers. The *C. glabrata* isolate (OD_{590}: 0.1-1.0) was a weak biofilm producer. Adding the MIC_{90} of DIO to yeast inhibited biofilm formation by 35.35–87.85% after 1 h of exposure, while the percentage inhibition of biofilm formation in cells treated with AFB ranged from 6.49–91.26%, as shown in Table 2; in most isolates, the inhibitory effect of DIO on biofilm formation was greater than that of AFB, as shown in Figure 3b. In *C. albicans* and *C. glabrata* isolates, no inhibitory effect on biofilm formation was evident from DIO or AFB. The effect of DIO and AFB was less evident in the inhibition of mature biofilms; as seen in Figure 3c, an inhibitory effect was only evident against mature biofilms of the isolates *C. tropicalis*, *C. krusei* and *C. auris*. The behavior of DIO and AFB was similar, with significant differences observed only with *C. auris*. The percentages of inhibition of mature biofilms by DIO and AFB on *Candida* spp., are shown in Table 3.

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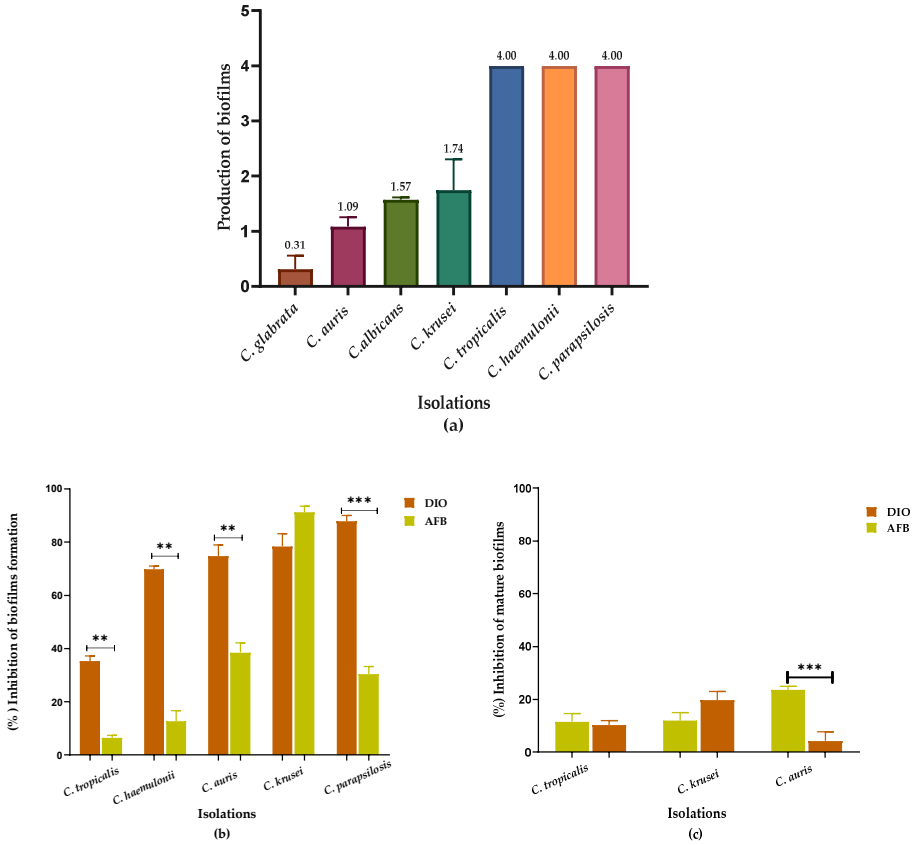

Figure 3. Action of DIO and AFB on *Candida* spp. biofilms; (a) biofilm formation at 37 °C for 28 h, where an OD_{590} > 3 indicates strong biomass production in the biofilms. (b) percentage of biofilm reduction after 1 h of treatment with MIC_{90} of DIO for each isolate and AFB (2 µg/mL). The results of the ANOVA (Brown-Forsythe test) with a value of *** $p < 0.001$ (*C. parapsilosis*) and ** $p < 0.01$ (*C. tropicalis*, *C. haemulonii*, *C. auris*) and the Games-Howell's post hoc test, with a confidence level of 95%, indicate that there are significant differences between the effect of DIO and the effect of AFB on biofilm inhibition in these isolates.

Table 2 shows the percentages of inhibition of DIO biofilm formation in *Candida* spp. during 24 hours of exposure.

Table 2. Percentages of inhibition of biofilm formation of DIO vs AFB in *Candida* spp.

Candida spp. isolates	DIO	AFB
C. albicans	00.00 ± 4.39	00.00 ± 4.48
C. glabrata	00.00 ± 6.63	00.00 ± 4.77
C. tropicalis	35.35 ± 1.88	6.49 ± 0.92
C. haemulonii	69.81 ± 1.16	12.75 ± 3.92
C. auris	74.75 ± 4.22	38.49 ± 3.71
C. krusei	78.41 ± 4.79	91.26 ± 2.17
C. parapsilosis	87.85 ± 2.18	30.43 ±2 .78

Table 3 shows the percentages of DIO inhibition against mature *Candida* spp. biofilms after 24 hours of incubation.

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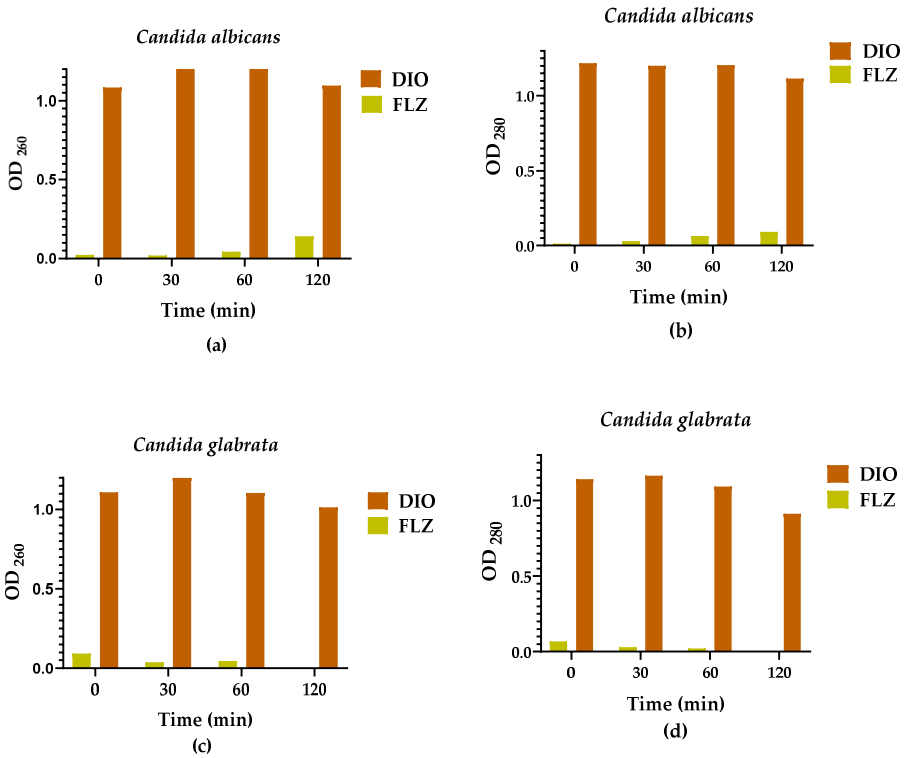
Table 3. Percentages of DIO inhibition against mature biofilms, DIO vs AFB in *Candida* spp.

Candida spp. isolates	DIO	AFB
C. albicans	00.00 ± 3.12	00.00 ± 3.14
C. glabrata	00.00 ± 5.68	00.00 ± 5.41
C. tropicalis	11.53 ± 3.09	10.2 ± 1.67
C. haemulonii	00.00 ± 0.00	00.00 ± 0.00
C. auris	23.65 ± 1.24	4.24 ± 3.48
C. krusei	11.92 ± 2.99	19.67 ± 3.32
C. parapsilosis	00.00 ± 0.00	00.00 ± 0.00

2.3. Leakage of Nucleic Acids and Proteins through the Fungal Membrane

The action of DIO on the membrane integrity of *Candida* spp. was evaluated by assays for the release of intracellular constituents that absorb at 260/280 nm, such as nucleic acids and proteins. These assays were performed at 0, 30, 60, and 120 min after treatment with the MIC_{90} of DIO for each isolate. As seen in Figure 4, OD_{260}/OD_{280} values in DIO-treated groups were significantly higher from baseline compared to FLZ treated groups, where minimal or even no (in some cases) release of intracellular material was observed in all *Candida* spp. isolates. These results indicate damage to fungal cell membrane permeability caused by DIO.



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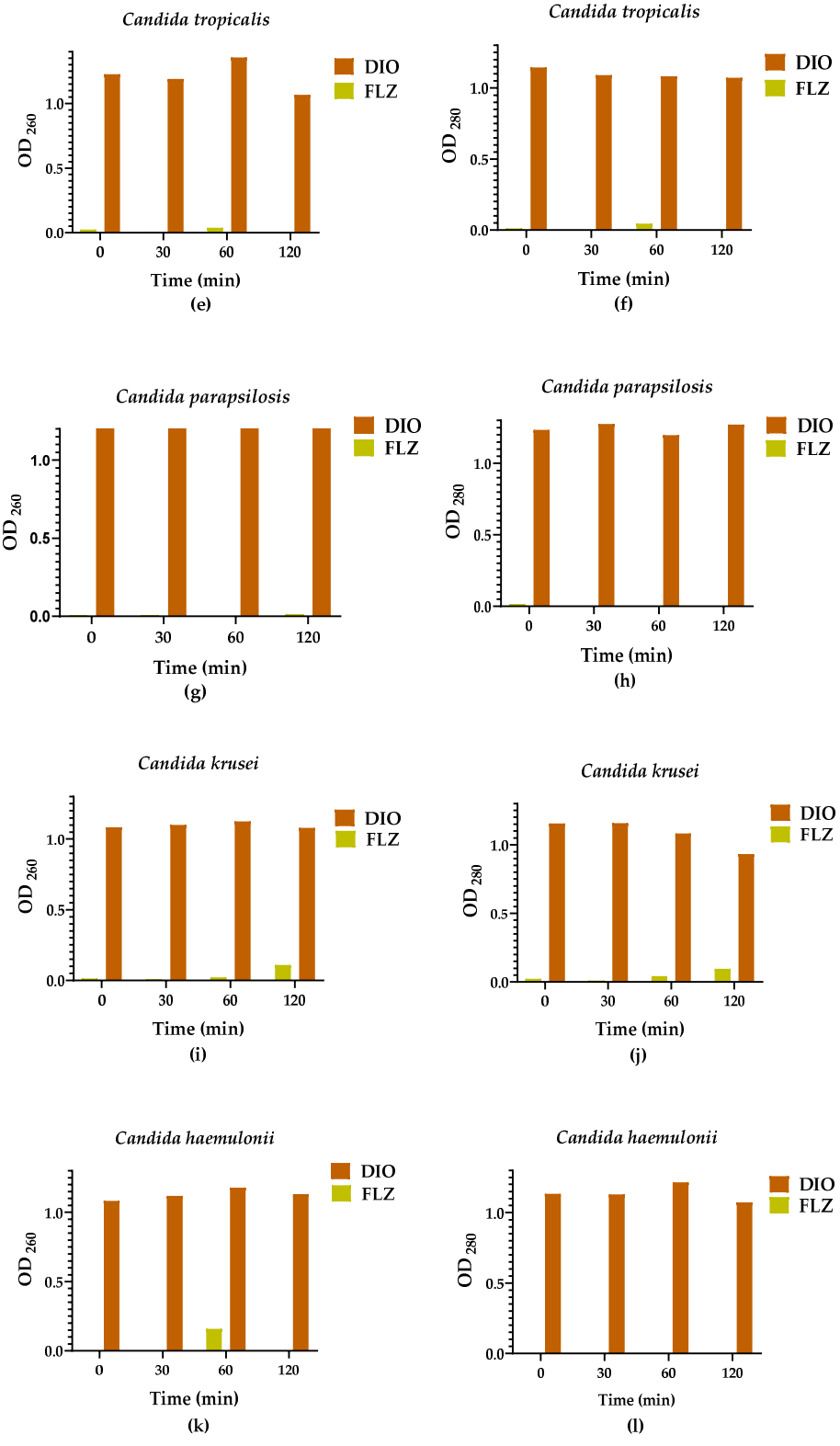

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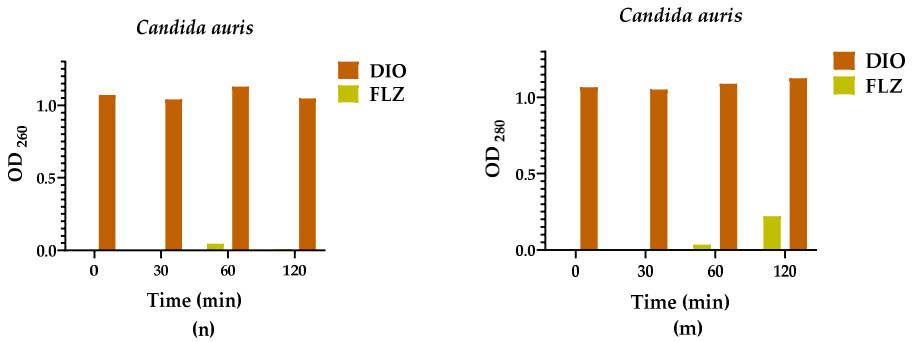

Figure 4. Intracellular content was released at 260/280 nm as a function of time, from *Candida* spp. treated with DIO (MIC_{90} µg/mL) and FLZ (MIC_{90} µg/mL). Results are expressed as the absorbance of the sample (treated with DIO) minus the absorbance of the control (samples without DIO). Analyses of variance (Brown Forsythe ANOVA test) revealed p values < 0.05 (260/280 nm), the Games-Howell´s post hoc test, with a 95% confidence level, showed significant differences between the effect of DIO and FLZ on the release of intracellular material from all *Candida* spp. isolates evaluated, demonstrating damage to the fungal membrane permeability caused by DIO.

2.4. Diosmin Action in Combination with Commercial Antifungals

Figure 5 shows the synergistic effect of DIO and the commercial antifungals FLZ and AFB against most of the *Candida* spp. isolates evaluated. The DIO-FLZ combination exhibited synergistic effects against four of the seven *Candida* spp. isolates tested (Ca02, Ct74, Cg01, and Cp02), while the DIO-AFB combination exhibited synergism against five of the seven strains tested (Ca02, Ct74, Cg01, Ch01, and Cau75). No interaction was observed against strain Ck01 in any of the tested combinations (DIO-FLZ and DIO-AFB). The DIO-FLZ combination did not interact against isolate Ch01; similarly, the DIO-AFB combination did not interact against isolate Cp02.

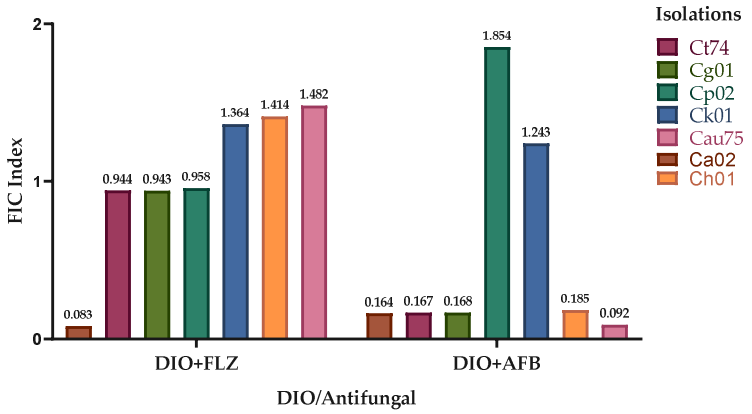

Figure 5. Effect of DIO in combination with FLZ and AFB against *Candida* spp. The fractional inhibitory concentration (FCI) indices of the different clinical isolates of *Candida* spp., are shown.

Table 4 shows that the minimum inhibitory concentrations (MIC_{90}) at which 90% of the yeasts were inhibited in synergistic activity were substantially lower than the individual MIC_{90} values of DIO and the antifungals used.

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Table 4. MIC_{90} values, individually and in combination, of DIO, FLZ and AFB against *Candida* spp.

Candida spp.	MIC_{90} Single			MIC_{90} in Combination		FIC Indices		Effect	
	FLZ	AFB	DIO	FLZ-DIO	AFB-DIO	FLZ-DIO	AFB-DIO	FLZ-DIO	AFB-DIO
Ca02	55.34	9.91	1150	2.2 - 49.9	0.82 - 93.99	0.08	0.16	Sng	Sng
Ct74	241.8	9.87	1526	112.6 – 730.6	1.18 - 73.32	0.94	0.17	Sng	Sng
Cg01	214.3	4.11	2251	103.5 - 1036	0.35 - 186.9	0.94	0.17	Sng	Sng
Ch01	133.9	8.89	1655	94.7 - 1170	0.82 - 153.5	1.41	0.18	S.I.	Sng
Ck01	69.99	3.30	2118	46.9 - 1469	2.08 - 1298	1.36	1.24	S.I.	S.I.
Cp02	42.09	4.05	1670	20.3 - 792.7	3.791- 1534	0.96	1.85	Sng	S.I.
Cau75	75.65	18.41	1786	55.4 - 1338	0.853- 82.89	1.48	0.09	S.I.	Sng

Sng: synergism; S.I.: no interaction.

3. Discussion

Candida spp. are pathogens of great global concern. These yeasts' remarkable ability to adapt to the hospital environment is primarily due to their multi drug resistance to conventional drugs used for their control and the expression of various virulence factors, including the production of powerful biofilms. This limits therapeutic options and increases morbidity and mortality rates, worsening the patient situation and increasing global financial costs. This situation sparks interest and drives the search for new molecules with antifungal properties. In this context, chemical compounds of natural or synthetic origin become an excellent alternative.

In this research we demonstrate that DIO, a flavonoid present in citrus fruits, has antifungal activity against clinical isolates of *Candida* spp., and this effect is concentration dependent. Results consistent with our study document the potential of DIO against methicillin resistant and methicillin sensitive *Staphylococcus aureus* (MRSA) strains; it has been shown that diosmetin (aglycone form of DIO) significantly suppresses the pharmacokinetic activity of MRSA in a dose-dependent manner. This could cause ATP deficiency and affect the bacterial efflux pump, which would contribute to the antibacterial action of diosmetin against MRSA [25,26]. Similar studies [22] reported the antibacterial effect of DIO against *P. aeruginosa* suggesting that this compound could be a potential candidate for the development of a new agent targeting *P. aeruginosa* infections by reducing its virulence mechanisms, showing a negative regulation of the associated genes (lasI and pvdS). DIO has been implicated in partial inhibition of ATP synthesis in *E. coli*, and DIO and diosmetin have been virtually coupled to the enzymes LdtMt1 and LdtMt2, which are involved in *M. tuberculosis* (Mtb) cell wall biosynthesis [21]. MIC and BMC antimicrobial assays have shown that pompia and lemon juices high in DIO and other flavonoids have inhibitory and antibiofilm effects against pathogenic bacteria including *P. aeruginosa*, *S. aureus* and *Enterococcus faecalis* [28]. The antiviral effect of DIO has also been documented [24], indicating that DIO significantly inhibited the varicella zoster virus (VZV). The antimicrobial effect of DIO has been poorly reported against bacteria, and the role of DIO in the treatment of infectious diseases such as malaria, dengue, chikungunya, SARS-CoV-2, and leishmaniasis may likely be investigated in the future [23]. This study reports for the first time the effect of DIO against clinical isolates of pathogenic yeasts *Candida* spp.

The damage to the permeability of the fungal cell membrane of *Candida* spp. caused by DIO was evidenced in the intracellular material leakage experiments (260/280 nm), which showed a significant and early release of intracellular material when the fungal cells were treated with DIO; these results are consistent with the results reported by [23], who showed antibacterial activity of DIO against

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strains of *E. coli*, *P. putida* and *S. aureus*, reporting that among the mechanisms of action of this compound is the alteration of the permeability of the cell membrane, as well as the formation of holes in the bacterial cell wall, the inhibition of transduction, the inhibition of respiratory enzymes due to the formation of free radicals and the inactivation of several enzymes. Our results suggest that damage to the cell membrane of *Candida* spp. is at least one of the mechanisms of antifungal action of this flavonoid.

Candida spp. have been widely documented for their ability to form potent biofilms [29], which increase resistance to both antifungal drugs and the host immune response, causing persistent infections; these biofilms vary depending on the source of the infection [30]. The *Candida* spp. isolates in this study were all biofilm producers (Figure 3a). We report the potential of DIO against fungal biofilms; this is in line with studies documented by [22], who recorded a high potential of DIO to disrupt both bacterial biofilm formation and eradication, including a significant reduction in biofilm biomass, exopolysaccharide and extracellular DNA production in *P. aeruginosa*, where treatment of the biofilm with DIO resulted in the lowest percentage of live microbial cells. When comparing the efficacy of DIO with AFB, we highlighted the role of DIO in inhibiting biofilm formation after 1 hour of treatment and in most cases it was significantly higher than with AFB (Figure 3b); however, the effect of DIO and AFB against mature biofilms was lower (Figure 3c), with similar effects; this is consistent with studies reported by [31], which indicate the ability of liposomal AFB to inhibit further biofilm growth, but its ineffectiveness in eradicating mature biofilms, even at high doses.

On the other hand, effective therapeutic regimens for the treatment of *Candida* spp. are limited. In this study, we highlight the synergistic effect of DIO in combination with the antifungals FLZ and AFB against antifungal resistant *Candida* spp. clinical isolates. We emphasize the synergistic effect of DIO-AFB against the multidrug resistant strain of *C. auris* Cau75, achieving substantial reductions in the MIC_{90} values of both treatments (Table 4). These results are consistent with studies demonstrating the synergistic effect of DIO and its aglycone form (diosmetin) with other drugs. The combination of DIO and amoxicillin-clavulanic acid has shown synergistic inhibition of mycobacterial growth, demonstrating greater mycobactericidal activity against *Mycobacterium marinum* [21,25]. Likewise, the synergistic effect of diosmetin with erythromycin against the ABC transporter overexpressed in MRSA has been documented [26]. Besides, the synergistic effects of DIO have also been reported against different types of cancer [32], revealing the synergistic chemotherapeutic effects of DIO in combination with BEZ-235 in the colorectal cancer cell line HCT-116. Likewise, the synergistic effect of DIO and interferon-α has been shown in metastatic pulmonary melanoma [33]. DIO in combination with other flavonoids has also shown a synergistic effect, in fact, hesperidin and DIO increased the cytotoxic activity of cisplatin on hepatocellular carcinoma [34]; likewise, DIO in combination with naringenin enhanced apoptosis in colon cancer cells [35]. Our results suggest that DIO could act as an important adjuvant in the treatment of *Candida* spp. multidrug resistant to antifungal drugs.

Our results provide important new information on the antifungal potential of the flavonoid DIO against *Candida* spp., demonstrating its action against the cell membrane and its inhibitory effect against fungal biofilms; in addition, its synergistic potential with the commercial antifungal FLZ. We also provide information that serves as a basis for future research to elucidate the antifungal mechanisms of action of this compound, which could serve as an adjuvant for the treatment of infections caused by these pathogenic yeasts. Importantly, plant derived compounds can potentially be used to combat multidrug resistant and biofilm forming strains of *Candida* spp., thus becoming a promising alternative to antifungal drugs.

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4. Materials and Methods

4.1. Reagents

The RPMI 1640, phosphate buffered saline (PBS), and yeast peptone dextrose broth (YPD) were obtained from Thermo Fisher Scientific, Waltham, MA, USA; 3-N-morpholinopropanesulfonic acid (MOPS) was obtained from Merck; potato dextrose broth (PDB), sabouraud dextrose agar (SDA), sabouraud dextrose broth (SDB), amphotericin B (AFB), crystal violet and Fluconazole (FLZ) used in this study were obtained from Sigma-Aldrich, USA; glacial acetic acid was obtained from Carlo Erba Reagents, Italy.

4.2. Diosmin (DIO)

Diosmin, 3′,5,7-trihydroxy-4′-methoxyflavone-7-rutinoside, ($C_{28}H_{32}O_{15}$) molecular weight: 608.54, was purchased from Sigma-Aldrich Inc, CAS number: 520-27-4.

4.3. Strains

Nine clinical isolates of *Candida* spp. including: *C. albicans* (Ca02 and Ca77), *C. glabrata* (Cg01), *C. tropicalis* (Ct03 and Ct74), *C. krusei* (Ck01), *C. haemulonii* (Ch01), *C. parapsilosis* (Cp02), and *C. auris* (Cau75) were used in this study. The isolates were cultured from the blood and urine culture samples of patients hospitalized at the Social Health Service S.A.S. in the city of Sincelejo, Colombia. All microorganisms were identified by standard methods: Vitek 2 Compact, Biomerieux SA, YST Vitek 2 Card and AST-YS08 Vitek 2 Card (Ref 420739). SDA medium and BBL CHROMagar Candida medium were used to maintain the cultures until the tests were carried out.

4.4. Antifungal Susceptibility Testing

The minimum inhibitory concentration (MIC) of DIO against clinical isolates of *Candida* spp., was defined as the lowest concentration at which 90% of fungal growth was inhibited (MIC_{90}) compared to the control. The MIC_{90} was determined by broth microdilution assays using 96 well microtiter plates (Nunclon Delta, Thermo Fisher Scientific, Waltham, MA, USA) as described in the *Clinical Laboratory Standards Institute* (CLSI) method (M27-A3) [36] and the *European Committee for Antimicrobial Susceptibility Testing* (EUCAST) method [37], with minor modifications. Serial dilutions were made in RPMI 1640 broth (pH 7.0) buffered with 0.165 M MOPS to obtain final concentrations of 3.90 to 1000 μg/mL of DIO in each reaction well. Stock solutions of DIO were prepared at 20000 μg/mL in DMSO and FLZ at 1500 μg/mL in 10% DMSO in distilled water. Assays were performed with a final volume of 200 μL per well as follows: 100 μL of fungal inoculum at a concentration of 1×10^6 CFU/mL and 100 μL of DIO adjusted to achieve the concentrations described above in a final reaction system. *Candida* spp. isolates without DIO and FLZ were used as growth controls and positive controls, respectively; wells with culture medium without inoculum and without DIO were used as negative controls. For each test, controls were run with different concentrations of DIO in culture medium without inoculum. The plates were incubated at 37 °C for 24 h. Inhibition of fungal growth by DIO was determined by the change in optical density using a SYNERGY LX microplate reader (Biotek), at 530 nm, from the start of incubation to the final time (24 h) [38], and the percentage reduction in growth was calculated using the following equation:

$$\%\text{Reduction} = (1 - (ODt_{24} - ODt_0/ODgc_{24} - ODgc_0)) \times 100$$

where, ODt_{24}: optical density of the test well at 24 h post-inoculation; ODt_0: optical density of the test well at 0 h post-inoculation; $ODgc_{24}$: optical density of the growth control well at 24 h post-inoculation; $ODgc_0$: optical density of the growth control well at 0 h post inoculation.

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4.5. Quantitative Evaluation of Biofilm Inhibition

Clinical isolates of *Candida* spp. were evaluated to quantify biofilm reduction in the presence of DIO following the methodology reported by [38], with some modifications. For biofilm formation, yeast colonies on SDA with 24 h of incubation were used to standardize the inoculum to a concentration of 1×10^6 cells/mL. Subsequently, 200 µL of the fungal inoculum was cultured in 96-well plates in YPD broth and incubated at 37 °C for 48 h. The broth was then removed from the microplates and 200 µL of the DIO MIC_{90} was added to each isolate in YPD broth, incubating at 37 °C for 1 h. Subsequently, floating cells were removed and the biofilm at the bottom of the wells was washed with deionized water three times. Six replicates of each sample were prepared. Cultures without DIO were used as a control and AFB as a positive control. Biofilm reduction was quantified by staining the wells with 0.1% crystal violet for 20 min. The samples were washed with deionized water until excess dye was removed. Finally, they were immersed in 250 µL of 30% glacial acetic acid. Absorbance values were measured at 590 nm (OD_{590}) using a SYNERGY LX microplate reader (Biotek). Biofilm production was grouped into the following categories: $OD_{590} < 0.1$: non-producers (NP), OD_{590} 0.1–1.0: weak producers (WP), OD_{590} 1.1–3.0: moderate producers (MP), and $OD_{590} > 3.0$: strong producers (SP). For biofilm inhibition assays, the standardized bacterial inoculum was incubated simultaneously with the MIC_{90} of DIO. Biofilm reduction was calculated using the following equation:

$$\% \text{ Biofilm reduction: } AbsCO - AbsDIO/AbsCO \times 100$$

where, AbsCO: absorbance of the control and AbsDIO: absorbance of the sample treated with DIO

4.6. Leakage of Nucleic Acids and Proteins through the Fungal Membrane

The release of intracellular material was measured according to the methodology proposed by [38], with some modifications. Yeasts grown in SDB were centrifuged at 3000× g for 20 min, washed three times and resuspended in 20 mL of PBS (pH 7.0). The cell suspension was then treated with DIO (MIC_{90} for each isolate) and incubated at 37 °C for 0, 30, 60 and 120 min. Subsequently, 2 mL of the samples were collected and centrifuged at 3000× g for 20 min. To determine the concentration of the released constituents, 2 mL of supernatant was used to measure the absorbance at 260/280 nm in a Spectroquant® Prove 300 UV/Vis spectrophotometer. Samples without DIO and samples with FLZ were used as controls. All assays were performed in triplicate.

4.7. Diosmin Action in Combination with Commercial Antifungals

To obtain the fractional inhibitory concentration indices (FICIs) of DIO in combination with FLZ and AFB against *Candida* spp., we followed the methodology proposed by [39]. Serial dilutions were made in RPMI 1640 broth containing 0.1% 2,3,5-triphenyltetrazolium chloride (CTT), reaching final concentrations in the range of 1000—3.9 µg/mL, 256—0.25 µg/mL and 32—0.062 µg/mL for DIO, FLZ and AFB, respectively. The assay was performed with a total volume of 200 µL per well, distributed as follows: 50 µL of DIO + 50 µL of FLZ and AFB were added to reach the concentrations described previously, as well as 100 µL of fungal inoculum at a concentration of 1×10^6 CFU/mL. Absorbance readings were measured with a Chromate 4300 ELISA reader at a wavelength of 630 nm, and subsequently, after 24 h of incubation at 37 °C, measurements were performed again. The FICI were calculated using the following equation:

FICIs = MIC_{90} DIO in combination/ MIC_{90} DIO single + MIC_{90} antifungal in combination/ MIC_{90} antifungal single

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The results were interpreted following the approach used in [39]. The FICIs are considered to have a synergistic effect (FIC index ≤ 1.0), a commutative effect (FIC index = 1), no interaction (1.0 < FIC index ≤ 2.0) or an antagonistic effect (FIC index > 2.0).

4.8. Statistical Analysis

The results were analyzed using GraphPad Prism software version 8.0 and Microsoft Excel version 2024. Initially, the Shapiro-Wilk test was used to determine the distribution of the data. Pearson's correlation coefficients were then used to measure the degree of linearity, the correlation between DIO concentration and the percentage reduction in fungal growth. To compare the effects of DIO and the antifungal agent AFB on biofilm reduction, Games-Howell's post hoc tests were used; this test was also used to compare the effects of DIO and the antifungal agent on intracellular membrane leakage (260/280 nm).

5. Conclusions

In this study, we investigated the antifungal potential of the flavonoid diosmin against clinical isolates of *Candida* spp., as well as its role in biofilm inhibition. We also explored its action against the cell membrane of these pathogens. We demonstrated the antifungal action of DIO against *Candida* spp. this effect being associated with damage to cell membrane integrity, in addition to its action against fungal biofilms. It is necessary to continue these studies, with the aim of elucidating the mechanisms of antifungal action of DIO, and its possible synergistic action with drugs, since this flavonoid shows promise as an alternative tool for the treatment and control of multi-resistant nosocomial pathogens such as *Candida* spp.

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