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EC PHARMACOLOGY AND TOXICOLOGY
Research Article

Antifungal Activity and Pharmacokinetic Prediction of Chalcone on Phospholipase-Producing *C. albicans* Isolates

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Received: June 20, 2020; **Published:** September 30, 2020

Abstract

Candida albicans is one of the major causative agents of human mycoses. Natural and synthetic chalcones have attracted attention due to a diversity of biological activities. We synthesized the 2,3-dyhydroxy-chalcone (DHC) and screened it for its antifungal activity against phospholipase-producing clinical isolates of *C. albicans*. DHC was further tested for its toxicity on oral mucosa keratinocyte cells (NOK cells) and *in vivo* in *Galleria mellonella*. The Minimum Inhibitory and Fungicidal Concentrations (MIC/MFC) of DHC and nystatin on *C. albicans* isolates and a reference strain were determined. The toxicity of DHC on oral NOK cells was assessed by the (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) method to determine the selectivity index (SI). The data were analyzed by ANOVA one-way with Tukey's test ($\alpha = 0.05$). DHC was active against phospholipase-producing *C. albicans* isolates, with MIC and MFC values ranging from 16.24 to 130.16 µM; and from 260.32 to 1041.31 µM, respectively. The MFC/MIC ratios observed suggested that DHC is mostly fungistatic. At MIC and higher concentrations, DHC showed low toxicity on NOK cells, and high selectivity for yeast cells. The compound tested at a concentration of up to 2000 µM did not present toxicity *in G. mellonella*. *In Silico* demonstrates that the DHC molecule has a desired profile when based on the Lipinski's and Veber's rules. Altogether, our study provides insights into the use of the newly modified 2,3-dyhydroxy chalcone as a promising candidate for the development of novel antifungal therapies.

Keywords: *Candida albicans; Clinical Isolate; 2,3-Dyhydroxy-Chalcone; Toxicity; Human Keratinocytes*

Introduction

Candida infections are of great concern in dentistry. Patients may present with infections superficial or indicative of systemic illness [1]. Several factors related to the host contribute to the beginning of the infection, including the use of orthodontic and prosthetic devices, changes in eating habits, poor oral hygiene, hormonal changes, immunosuppression, radiation and chemotherapy. Furthermore, factors

related to microorganisms also represent an important role, such as adhesion to host tissues, the ability to form biofilms and the production of hydrolytic enzymes [2,3].

A number of studies have demonstrated a relationship between *Candida* spp. carriage and periodontal disease with prevalence 40% [4-9]. The ability of *C. albicans* to produce extracellular hydrolytic enzymes, such as phospholipases and proteases, which play an important role in yeast adhesion to, and tissue invasion of, host cells, may favor the inflammatory process [10,11].

Due to the widespread use of antifungal agents, prolonged or intermittent treatment, an increase in the frequency of resistant *Candida* spp. isolates has been observed. Such microbial resistance to various drugs has encouraged the search for novel compounds with different mechanisms and more potent antifungal activity, including those of natural origin [12,13].

Chalcones are α-β-insaturated ketones present in several plant species. These compounds have been used as precursors for biosynthetic route of flavonoids. Synthesis of chalcones may be accomplished by Claisen-Schmitd Condensation between the ketone and aldehyde using acid and basic catalysis [14]. In recent years, natural and synthetic chalcones have attracted attention due to a diversity of biological activities such as antimalarial, anti-leishmania, anti-HIV, anti-fungal, anti-tuberculosis, among others [15-21].

In this study, the 2,3-dyhydroxy-chalcone (DHC) was synthesized and tested for its antifungal activity against phospholipase-producing clinical isolates of *Candida albicans* from patients with periodontitis and type II diabetes mellitus. To provide preliminary toxicological evidence, we further tested DHC for its toxicity on oral mucosa keratinocyte cells (NOK). Our findings suggest that DHC might be a promising alternative candidate for the treatment of fungal infections in systemically susceptible individuals.

Materials and Methods

Synthesis of 2,3-dyhydroxy-chalcone

The 2,3-dyhydroxy-chalcone (DHC) (Figure 1) was synthetized following previous methods [14]. The following parameters were obtained: (*E*) 2,3-dyhydroxy-chalcone. Yellow solid, 43% yield. ^{1}H NMR (500 MHz, DMSO-d_6), δ 6.86 (dd; J = 1.5 and 7.5 Hz, H-4), δ 6.70 (dd; J = 7.5 and 7.5 Hz, H-5), δ 7.31 (dd; J = 1.5 and 7.5 Hz, H-6), δ 8.07 (dd; J = 1.5 and 7.5 Hz, H-2' and H-6'), δ 7.55 (dd; J = 7.5 and 7.5 Hz, H-3' and H-5'), δ 7.63 (dd; J = 1.5 and 7.5 Hz, H-4'), δ 8.06 (d; J = 15.5 Hz, H-α'), δ 7.77 (d; J = 15.5 Hz, H-β'), δ 9.70 (s; 2-OH), δ 9.13 (s; 3-OH). ^{13}C NMR (500 MHz, DMSO-d_6), δ 118.7 (C-1), 145.9 (C-2), 145.7 (C-3), 117.1 (C-4), 121.9 (C-5), 119.1 (C-6), 137.9 C-1'), 128.7 (C-2' and C-6'), 128.2 (C-3' and C-5'), 132.8 (C-4'), 120.9 (C-α), 139.9 (C-β), 189.6 (C-β').

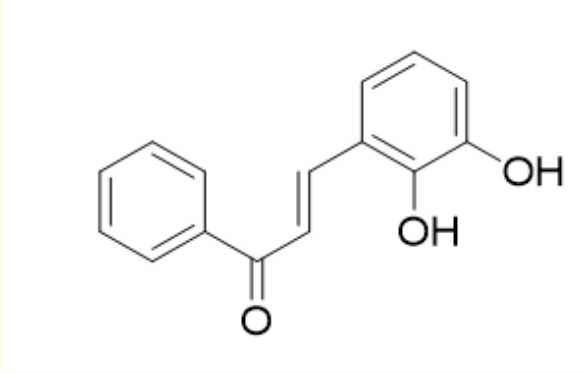

Figure 1: *Molecular structure of 2,3-dyhydroxy-chalcone.*

Fungal strains and growth conditions

This study was approved by the Research Ethics Committee at Piracicaba Dental School, University of Campinas (FOP/UNICAMP), under protocol no. 062/2008. Previously, nine clinical isolates of *Candida albicans* were obtained from patients with periodontitis and type II diabetes mellitus. These isolates were shown to produce high concentrations of phospholipases [8]. DHC was tested herein for its antifungal activity against these isolates and the reference strain *C. albicans* ATCC 90028. The strains were maintained as frozen stocks at -80°C until use. For the assays, the strains were subcultured onto Sabouraud Dextrose Broth (SDB) (Difco®, Detroit, MI, USA) and a single colony was inoculated into SDB medium and incubated at 37°C for 24h.

Determination of the antifungal activity

The Minimum Inhibitory Concentration of DHC against the clinical isolates and reference strain was determined according to the protocol M27-S3 of the Clinical and Laboratory Standards Institute (CLSI), as previously described [22]. The chalcone derivative was solubilized in DMSO to obtain stock and working solutions. Serial dilutions of the chalcone (1.99 to 1041.31 mM) and standard drug nystatin (0.13 to 17.26 mM) in RPMI-1640 (Sigma-Aldrich, St. Louis, MO, USA) were obtained in 96-well plates. A hundred microliter aliquots of fungal inoculum were added to each well to a final concentration of 1 x 10^4 CFU/mL. The plates were incubated at 37°C under shaking (150 rpm) for 24 h for determination of the Minimum Inhibitory Concentration (MIC). The Minimum Fungicidal Concentration (MFC) of DHC against clinical isolates of *C. albicans* was determined by subculturing an aliquot from each well onto Sabouraud Dextrose Agar. After 24h, the MFC was determined as the lowest concentration of the chalcone able to inhibit visible cell growth on the solid medium.

Cytotoxic effects on human keratinocytes

The toxicity of DHC on oral mucosa keratinocytes (NOK cells) was assessed by the MTT assay [23]. The chalcone derivative was diluted in Keratinocyte-SFM medium (1x) (Gibco) and tested at ten different concentrations ranging from 1.99 to 1041.31 mM. Cells were treated for 24h and then processed for cell viability measurement using the MTT salt (5 mg/mL) (Sigma-Aldrich, St. Louis, MO, USA) followed by spectrophotometric reading at 560 nm. The percentage of viable cells was calculated for each DHC concentration tested.

In Silico drug-likeness and pharmacokinetics investigations

In order to assess *in silico* drug-likeness and pharmacokinetics properties of DHC, we performed investigation using Molinspiration and PreADMET toolkits, respectively. Parameters were used according to Lipinski's and Veber's rules, including values of weight molecular (WM), number of hydrogen bond acceptors (HBA), number of hydrogen bond donors (HBD), topological polar surface area (TPSA), logarithm of compound partition coefficient between n-octanol and water (log Po/w) number of routable bonds (NROTB), molecular volume (Å3), percentage of human intestinal absorption (% HIA) and blood-brain barrier (BBB) penetration [24,25].

Statistical analysis

All assays were performed in triplicate of three independent experiments. The data were analyzed by ANOVA with Tukey's pairwise comparison test, with a 5% significance level. GraphPad Prism 5.0 software (San Diego, CA, USA) was used for data analysis.

Results

DHC has antifungal activity *in vitro*

The Minimum Inhibitory and Fungicidal Concentrations (MIC/MFC) of DHC and nystatin (standard-drug) on *C. albicans* isolates and reference strain are listed in table 1. DHC was found to be active against phospholipase-producing clinical isolates of *Candida albicans,* with MIC values ranging between 16.24 and 130.16 mM. Isolate 1 was the most sensitive to treatment with DHC, followed by isolates 3 and 9, and 2, 5, 6, 7 and 8. Isolate 4 proved to be the least sensitive to DHC, with a higher MIC value. The MFC of DHC on clinical isolates ranged between 260.32 and 1041.31 mM. The MFC/MIC ratio was calculated to determine if the extracts have fungistatic (MFC/MIC ≥ 4) or fungicidal (MFC/MIC < 4) properties. MFC/MIC ratios above 4 suggested that DHC is mostly fungistatic on these clinical isolates.

C. albicans Strain	2,3-dyhydroxy-chalcone			Nystatin			
	MIC (µM)	MFC (µM)	SI (selectivity index)	MFC/MIC Ratio	MIC (µM)	MFC (µM)	SI (selectivity index)
Isolate 1	16.27	32.54	128.0	2	1.07	2.15	93.4
Isolate 2	65.08	520.65	32.0	8	2.15	8.63	46.5
Isolate 3	32.54	260.32	64.0	8	1.07	2.15	93.4
Isolate 4	130.16	260.32	16.0	2	2.15	4.31	46,5
Isolate 5	65.08	260.32	32.0	7	1.07	2.15	93.4
Isolate 6	65.08	260.32	32.0	7	1.07	2.15	93.4
Isolate 7	65.08	260.32	32.0	7	1.07	2.15	93.4
Isolate 8	65.08	260.32	32.0	7	1.07	1.07	93.4
Isolate 9	32.54	260.32	64.0	8	2.15	4.31	46.5
ATCC 90028	16.27	32.54	128.0	2	1.07	2.15	93.4

Table 1: *Minimum Inhibitory and Fungicidal Concentrations (MIC/MFC) and Selectivity Index of 2,3-dyhydroxy-chalcone and nystatin (standard-drug) on C. albicans isolates and reference strain.*

**Fungistatic (MFC/MIC ≥ 4) or fungicidal (MFC/MIC < 4) activity.*

DHC has low toxicity on human keratinocytes

The chalcone derivative was evaluated for its cytotoxicity on oral mucosa keratinocytes cells (NOK). At MICs and higher concentrations, DHC showed low toxicity on NOK cells, as shown in figure 2. Cells treated with DHC at MIC (16.24 to 130.16 µM) showed viability rates between 80% and 67%, respectively. In addition, DHC showed high selectivity for *C. albicans* over NOK cells, with selectivity index (SI) values ranging from 16 to 128. Nystatin presented selectivity index (SI) values ranging from 46 to 93.

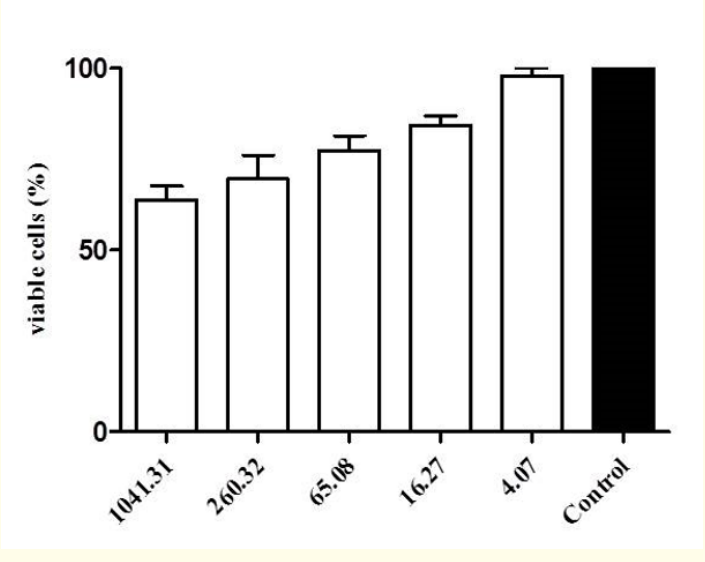

Figure 2: *Effects of 2,3-dyhydroxy-chalcone on the viability of oral mucosal keratinocytes by the MTT method. The data are presented as percentage of living cells upon treatment with different concentrations of the compound. One way analysis of variance with Tukey's post-hoc test ($p < 0.0001$).*

DHC - *In silico* drug-likeness and pharmacokinetics investigations

The results presented for the DHC *in silico* based on the rules of Lipinski and Veber were logarithm of compound partition coefficient between n-octanol and water (log Pow / w = 2.87), weight molecular (WM = 240.26), number of hydrogen bond donors (HBD = 2) and number of hydrogen bond acceptors (HBA = 3), number of routable bonds (NROTB = 3), topological polar surface area (TPSA = 57.53), molecular volume (Å3 = 217.89), percentage of human intestinal absorption (HIA = 92.52), blood-brain barrier penetration (BBB = 2.0), $CaCO_2$ cells (16.21). These results demonstrate that the DHC molecule has a desired profile when based on the Lipinski's and Veber's rules.

Table 2 shows *in silico* drug-likeness and pharmacokinetics predictions.

Entry	$logP_{o/w}$	MW (Da)	HBD	HBA	Lipinsk's Violations	NROBT	Veber's Violations	TPSA (Å^2)	Volume (Å3)	HIA (%)	BBB	$CaCO_2$
2,3-dyhydroxy-chalcone	2.87	240.26	2	3	0	3	0	57.53	217.89	92.52	2.00	16.21
log $P_{o/w}$ = Logarithm of compound partition coefficient between *n*-octanol and water; WM = Weight molecular, HBD = Number of hydrogen bond donors; HBA = Number of hydrogen bond acceptors; NROTB = Number of routable bonds, TPSA = Topological polar surface area; Volume = Molecular volume (Å^3); HIA = Percentage of human intestinal absorption; BBB = Blood-brain barrier penetration												

Table 2: *In silico drug-likeness and pharmacokinetics predictions.*

Discussion

Yeasts are considered important causative agents of a wide spectrum of infections, ranging from skin and mucocutaneous infections to life-threatening, invasive conditions. While the susceptibility of collection *Candida* strains to standard antifungal drugs is relatively known, the study of clinical isolates that may not follow the same virulence-related phenotypes, is critical [3,26-28]. The limited number of antifungal drug classes has encouraged the discovery and development of novel drugs derived from naturally-occurring agents [13,29]. Much research has been dedicated to explore the therapeutic values of chalcone derivatives [16]. Chalcones are compounds classified as open chain phenols, from the family of flavonoids, derived from plants and present structural diversity in the plant kingdom and have also a great variety of biological activities due to the presence of many physiologically active compounds. In addition, chalcones are important substrates for the accomplishment of several organic synthesis reactions [30-32]. Our results demonstrated that 2,3-dyhydroxy-chalcone has an excellent antifungal potential, as well as low toxicity on oral cells and high selectivity for *C. albicans* cells from individuals with periodontitis and diabetes. The strains this study were isolated from eleven patients with a clinical diagnosis of generalized chronic periodontitis and medical diagnoses of type 2 diabetes mellitus. These colonies showed high capacity to produce phospholipase, proteinase and hemolysis [8,10]. Despite the high virulence presented, they were susceptible to the compound used.

The cellular wall of fungi and the enzymes involved in biosynthesis and assembly of their components are considered potential targets of compounds with antifungal activity, as the cell wall is an essential structure for fungi and it is absent in mammalian cells. Inhibitors of the biosynthesis of β-glucan, chitin and mannoproteins of the components of the fungal cell wall are therefore interesting candidates for pharmaceutical application [33]. Lopes., *et al*. [34] demonstrated that chalcones with different substituents on rings A and B have antifungal activity against dermatophytes. According to this study, some of the chalcone derivatives and analogs act inhibiting the biosynthesis of one or both polymers of the cell wall. Several applications of chalcones and their derivatives have encouraged researchers to improve and modify their synthesis as an alternative approach for the treatment of pathogenic bacterial and fungal infections [15]. Three novel series of chalcone derivatives containing an aminoguanidine or acylhydrazone moiety were designed, synthesized and evaluated for their antibacterial, antifungal and anti-inflammatory activities [35]. Most of the synthesized compounds showed potent inhibitory activity on various bacteria and fungus, with MICs ranging from 1.0 to 8.0 μg/mL. In our study, DHC was found to be active against phospholipase-producing clinical isolates of *C. albicans* at concentrations as low as 16.24 μM.

Batovska., *et al*. [36] studied 44 chalcones and found that all were able to moderately inhibit *C. albicans* growth, with MICs ranging from 31.0 to 62.5 μg/mL. According to these authors, activity on *C. albicans* is dependent on the ability of the chalcone molecule to interact with the sulfhydryl group of the yeast cell membrane proteins. Other study showed that ring A substitution did not influence the activity potential of the chalcone, and that ring B substitution of chalcones modulate their activity on *C. albicans* [35]. The antifungal activity may be correlated with the position of the hydroxyl substituent on the chalcone ring B [36]. In addition to antifungal activity chalcones have also been reported to possess antimalarial, anti-leishmania, anti-HIV, anti-tumor, anti-tuberculosis and tyrosine kinase inhibitor properties [15,18-21,37,38]. There have been reports of antifungal activity of chalcones against *Paracoccidaides brasiliensis* and *Cryptococcus gattii*. Antitumor activity was also observed against UACC-62 (melanoma), MCF-7 (adenocarcinoma) and TK-10 (renal carcinoma) cells [39].

Pharmacokinetics properties of compounds interfere significantly with efficacy, oral absorption and adverse reactions in animal and humans [40]. Almost 30% of the drugs fail during development because of pharmacokinetic problems such as inefficient absorption, distribution, metabolism or excretion [41]. Thus, a compound which exhibits lower molecular flexibility, low polar surface area, low donor counts and hydrogen acceptors are important predictors of good pharmacokinetics. In addition, the permeability and bioavailability of the compounds are also associated with some basic molecular descriptors, such as logP (partition coefficient), molecular weight (MW) [41].

Prediction of human intestinal absorption (HIA) is a major goal in the design, optimization, and selection of candidates for development as oral drugs. The growth in drug discovery of combinatorial chemistry methods, where large numbers of candidate compounds are synthesized and screened in parallel for *in vitro* pharmacological activity, has dramatically increased the demand for rapid and efficient models for estimating HIA and other biopharmaceutical properties. *In vivo* animal studies have long been used, but these models are costly and labor intensive, have low throughput, and consume large amounts of test sample [42,43]. Yee classified intestinal absorption of compounds according to %HIA values being that from 70 to 100% is considered a good absorption corroborating with our results [44].

Moreover, blood-brain barrier (BBB) is a self-defense mechanism, controlling passage of compounds from blood to brain. This is very important and should be evaluated mainly if the pathogen is in the blood-brain barrier. Studies performed to Ma and co-authors (2005), demonstrate that high absorption to CNS occur when is more than 2.0; moderate absorption in the range 2.0 - 0.1 and poor absorption is less than 0.1 [45].

In this context, Lipinski and co-authors established drug-likeness rules: log Po/w ≤ 5.00, MW ≤ 500.00 Da, HBD ≤ 5.00 and HBA ≤ 10. For this author, compounds which do not violate more than one of these rules, may be a good drug candidate [46]. DHC presented log P ≤ 2.87, MW ≤ 240.26 Da, HBD ≤ 2 and HBA ≤ 3, do not violate any rule. At same form, Veber and co-authors suggested two central drug-

likeness properties: HBA + HBD ≤ 12 and NROBT ≤ 10 [47]. DHC can be present a good properties to choose molecules for drugs development (Table 2). Thus, this molecule has antifungal effect and low toxicity.

Conclusion

Altogether, our study provides insights into the use of the newly modified 2,3-dyhydroxy chalcone as a promising candidate for the development of novel antifungal therapies. Further research should determine its precise mechanism of action in the yeast cell and toxicity in other biologically relevant models.

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