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► To cite this version:

Diego Garcia Miranda, Lucas de Paula Ramos, Flavia Villaça Morais, Letícia Silva Nascimento, Isadora Abdalla Ferreira, et al.. Antifungal and Cytocompatible Properties of Juglans regia Extract for Dental Applications: A Novel Approach Against Oral Candida Infections. Applied Sciences, 2025, 15 (19), pp.10531. <10.3390/app151910531>. <hal-05289454>

HAL Id: hal-05289454

<https://hal.science/hal-05289454v1>

Submitted on 29 Sep 2025

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Article

Antifungal and Cytocompatible Properties of *Juglans regia* Extract for Dental Applications: A Novel Approach Against Oral *Candida* Infections

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Academic Editor: Maria Pia Ferraz

Received: 3 September 2025

Revised: 23 September 2025

Accepted: 28 September 2025

Published: 29 September 2025

Citation: Miranda, D.G.; Ramos, L.d.P.; Moraes, F.V.; Nascimento, L.S.; Ferreira, I.A.; Guimarães, B.M.; Tomé, F.M.; Rodrigues, F.P.; Carrouel, F. Antifungal and Cytocompatible Properties of *Juglans regia* Extract for Dental Applications: A Novel Approach Against Oral *Candida* Infections. *Appl. Sci.* **2025**, *15*, 10531. <https://doi.org/10.3390/app151910531>

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Featured Application

This study addresses the preclinical analysis of the use of *Juglans regia* glycolic extract as a possible pharmacological agent in dental materials to combat oral candidiasis.

Abstract

Oral *Candida* infections result from the overgrowth of this opportunistic fungus in the oral mucosa. Risk factors include immunosuppression, antibiotic or corticosteroid use, xerostomia, and conditions such as diabetes mellitus. Fungal resistance in *Candida* spp. has become a significant challenge, especially due to the excessive use of conventional antifungals such as azoles, echinocandins, and polyenes. Therefore, this study aims to determine the spectrum of antifungal activity of *Juglans regia* and assess its cytotoxicity on hepatocytes. Thus, a broth microdilution test was conducted according to the CLSI (M27-A3) guidelines. After initial screening, biofilm tests were conducted using the crystal violet (CV) and metabolic activity assays (MTT). Cytotoxicity was evaluated on human hepatocytes (HEPG2). The *J. regia* extract showed dose-dependent antifungal activity. At a concentration of 200 mg/mL, inhibition was greater according to the CV test in *Candida albicans* (31%) and *Candida tropicalis* (30.4%), while the MTT assay indicated a greater reduction in viability in *C. albicans* (61%) and *C. glabrata* (53.5%). At 100 mg/mL, *C. albicans* remained sensitive (37.7% CV; 71.6% MTT), while *C. krusei* and *C. dubliniensis* showed low viability by MTT (18.4% and 11.8%, respectively). At 50 mg/mL, *C. albicans* remained affected (74.3% MTT), but *C. krusei*, *C. dubliniensis*, and *C. guilliermondii* showed the lowest

viability values ($\leq 19.4\%$ MTT), suggesting greater sensitivity to lower concentrations. These results indicate variation in susceptibility between species, with *C. albicans* being consistently inhibited, while *C. krusei* and *C. dubliniensis* responded better to lower doses. The extract showed cytocompatibility when applied to human hepatoma cells (HEPG2) and therefore holds significant potential for developing a new therapeutic approach.

Keywords: antifungal agents; herbal medicine; *Candida* spp.; *Juglans regia*

1. Introduction

Antifungal resistance has emerged as a major global public health challenge, with increasing prevalence and diverse underlying mechanisms. The multifactorial problem includes genetic mutations in drug targets (such as ERG11, a gene encoding lanosterol 14 α -demethylase, an enzyme involved in ergosterol biosynthesis in *Candida* yeasts), overexpression of drug efflux via ABC and MFS transporters, and alterations in the composition and integrity of the fungal cell membrane [1,2].

The problem is further aggravated by the indiscriminate use of antifungals in both clinical and agricultural settings, while the therapeutic arsenal remains limited and the development of new drug classes progresses slowly [1–3]. Oral candidiasis is more than a local discomfort; it may impair feeding, reduce quality of life, and serve as an early indicator of systemic disease. In severe cases, it increases the risk of invasive candidiasis, a life-threatening infection associated with high morbidity and mortality [4]. Current therapies rely heavily on azoles and echinocandins, but resistance is increasingly reported, especially in *Candida glabrata* and emerging multidrug-resistant pathogens such as *Candida auris* [5–8]. These trends highlight the urgent need for alternative, safe, and effective antifungal strategies tailored to oral health.

In this context, phytotherapy offers promising opportunities. Natural products are known to provide diverse bioactive compounds with antimicrobial properties, often with fewer side effects and lower risks of resistance development compared with synthetic drugs [1,9]. *Juglans regia* (Walnut), belonging to the *Juglandaceae* family and traditionally used in folk medicine, represents a particularly interesting candidate. Extracts from its leaves, bark, and fruits are rich in polyphenols and flavonoids such as quercetin, myricetin, and avicularin, compounds with documented antimicrobial and antioxidant activities [10–20]. In traditional dental hygiene practices, especially in South Asia, walnut bark and leaves have been used for oral cleaning and to alleviate gum disease [14,17].

Recent studies have confirmed the antimicrobial potential of *J. regia* against various bacterial and fungal species, including drug-resistant strains. For example, Sytykiewicz et al. [18] demonstrated that methanolic and aqueous extracts prepared from *J. regia* leaves exhibited significant antifungal activity against *Candida albicans* isolates. Raja et al. [19] further reinforced the anti-*Candida* activity of the ethanolic root extract, which caused marked morphological alterations in yeast cells and suppressed key virulence factors essential for pathogenicity. In addition, Jafer and Naser [20] reported that both aqueous and methanolic extracts displayed antimicrobial activity against pathogenic yeasts and bacteria, with the methanolic extract consistently more potent across all tested microorganisms. These examples highlight the broad antifungal potential of *J. regia* and support further exploration of its pharmacological properties.

Thus, the objective of this study was to evaluate the antifungal activity of a glycolic extract of *J. regia* against six clinically relevant *Candida* species, as well as to evaluate its cytocompatibility on human hepatoma cells (HEPG2). The overarching objective was to

explore its potential as a novel antifungal agent for dental applications, particularly in preventive and therapeutic formulations targeting oral candidiasis.

2. Materials and Methods

2.1. Chemical Reagents

Phosphate buffer saline (PBS) (code: P2272, Sigma-Aldrich[®], St. Louis, MO, USA), Methanol (CAS n^o: 67-56-1, purity: 99.8%, Synth[®], Diadema, Brazil), RPMI 1640 medium with glutamine, without bicarbonate and phenol red indicator (Himedia, Mumbai, India), MOPS [3-(N-morpholino) propane sulfonic acid] pH 7.0 ± 0.1 (Sigma-Aldrich, St. Louis, MO, USA), Sabouraud Dextrose agar (Difco Laboratories, Detroit, MI, USA), Violet Crystal (CV) (1% *v/v*) (Synth, Diadema, Brazil), acetic acid (Synth, Diadema, Brazil), Eagle's medium modified by Dulbecco (DMEM; LGC Biotechnology[®], Cotia, Brazil), Fetal Bovine Serum (FBS; Invitrogen[®], New York, NY, USA), 7-Hidróxi-3H-fenoxazin-3-ona-10-óxido (Resazurin) (CAS n^o: 62758-13-8, code: R1017, Sigma-Aldrich[®], St. Louis, MO, USA), and ethanol (CAS n^o: 64-17-5, purity:99.5%, Synth[®], Diadema, Brazil) were used in this study.

2.2. Equipment

A Class II biological safety cabinet (Veco[®], biosseg-06, Sumaré, São Paulo, Brazil), analytical balance (Mettler Toledo[®], Balance XPR106DUH/A, Columbus, OH, USA), type I ultrapure water purification system (Allcron[®], direct-Pure[®] Genie, São Paulo, Brazil), autoclave (Cristofoli Biossegurança[®], Vitale21, Campo Mourão, Parana, Brazil), Phmeter (Digimed[®], DM-20, São Paulo, Brazil), CO₂ incubator (Sanyo[®], MCO-19AIC(UV), Osaka, Japan), water bath precision (TermoFisher Scientific[®], TSGP02, Waltham, MA, USA), refrigerated centrifuge (Labnet[®], HEREMLE Z300[®], Madrid, Spain), inverted microscope (Zeiss[®], Axiovert 40C, Jena, Thuringia, Germany), and spectrophotometer (Lonza Biotek[®], ELX808LBS, Winooski, VT, USA) were used in this study.

2.3. Plant Extract

Glycolic extract of *J. regia* (Walnut) (CAS n^o: 84012-43-1; lot: PRODO18746, Mapric Greentech Company[®], São Paulo, Brazil) was created by percolation using a mixture of 20% (*w/w*) plant leaf material. All physical–chemical characterizations (HPLC and MALDI-TOF) were reported in Miranda et al. [15], revealing the presence of three major compounds: Gallic acid, Regiolona, and Miquelianina, detected at *m/z* 170.12, 231.25, and 440.1, respectively.

2.4. Fungal Strains

Antifungal activity was evaluated with the following reference strains (ATCC—American Type Culture Collection): *Candida albicans* (ATCC 18804), *Candida dubliniensis* (ATCC MYA 646), *Candida glabrata* (ATCC 9030), *Candida guilhermondii* (ATCC 6260), *Candida krusei* (ATCC 6258), and *Candida tropicalis* (ATCC 13803). The strains were seeded on Sabouraud dextrose agar for reactivation in a bacteriological incubator at 37 °C and kept for 24 h.

2.5. Antimicrobial Activity in Planktonic Cultures

The minimum inhibitory concentration (MIC) and minimum microbicidal concentration (MMC) of the extract were determined by the broth microdilution method, according to the Clinical and Laboratory Standards Institute guidelines (CLSI—M27-A3 for yeast). This inoculum was prepared from the cultures incubated at 37 °C for 24 h in a sterile physiological solution (0.9% NaCl) and standardized to 10⁶ CFU/mL. Subsequently, 10 dilutions of the extract were prepared in 96-well microplates using RPMI 1640 medium buffered with MOPS for yeasts, generating values of 100, 50, 25, 12.5, 6.25, 3.12, 1.56, 0.78, 0.39,

and 0.19 mg/mL of the extract. Then, 100 µL of the standardized inoculum was added in each well, followed by 24 h of incubation. MIC was determined at the last well showing no turbidity. MMC was determined by inoculating 10 µL of wells aliquots on Sabouraud Dextrose agar for yeast.

2.6. Synthesis of Biofilm Matrix

Biofilms were performed in 96-well microplates from standard suspensions containing 10^7 CFU/mL incubated at 37 °C under agitation at 75 rpm for 48 h, with medium replacement after 24 h. After incubation, *J. regia* extract was placed in contact with the biofilms. Nystatin 100,000 IU/mL was used as the positive control, and sterile saline (0.9% NaCl) was used as the negative control. For all treatments, biofilms were exposed to the extract for 5 min, and the final volume in each well was 200 µL. Subsequently, solutions were discarded, and biofilm was washed twice with sterile saline (0.9% NaCl) [21,22].

2.7. Crystal Violet (CV) Staining

Biofilms were fixed with 200 µL of methanol per 20 min. Then, the solution was removed, and the plate was incubated at 37 °C for 24 h for drying. Crystal violet (1% v/v) was added for 5 min, and then the dye was removed. The wells were washed with sterile physiological solution (NaCl 0.9%) and 33% acetic acid. The plate was read in a microplate reader at a wavelength of 570 nm, and optical densities (ODs) were converted to biofilm biomass [21].

2.8. Metabolic Activity of Microorganisms by MTT Assay

MTT solution (0.5 mg/mL, 100 µL/well) was added to biofilms and incubated for 1 h at 37 °C (Sigma Aldrich, Saint Louis, MI, USA). After incubation, the solution was removed, and 200 µL of dimethyl sulfoxide (DMSO; Sigma Aldrich, Saint Louis, MI, USA) was added into the wells. The plates were incubated again at 37 °C for 10 min and placed on the stirrer (Micro plate shaker MIX-1500, Miulab, Hangzhou, China), under constant agitation for 10 min. The plates were read on a microplate reader (Lonza Biotek ELX808LBS, Winooski, VT, USA) at a wavelength of 570 nm, and ODs were converted to the metabolic activity of the microorganism. Cell viability was expressed as a percentage of the untreated control, which was set to 100% [22].

2.9. Cell Viability by Resazurin Assay

Human hepatoma cells (HEPG2; obtained from the Bank of Cells of Rio de Janeiro, Association Scientific Technical Paul Ehrlich (APABCAM—RJ)) were cultured in Dulbecco's Modified Eagle's Medium supplemented with 10% FBS, incubated at 37 °C and 5% CO₂. The cytotoxicity assay was performed with 4×10^4 viable cells, plated on a 96-well microplate, and incubated (37 °C and 5% CO₂) for 24 h. After cell adhesion, the supernatant was removed to apply *J. regia* extract at concentrations of 50, 100, and 200 mg/mL for 5 min. The control group received only DMEM + 10% FBS treatment for the same period. Each experimental group was performed with n = 10 and two independent repetitions. Afterwards, the wells were washed twice with sterile saline (0.9% NaCl). Then, the plates were used for the cell viability test.

Metabolic activity was assessed using resazurin at 440 µM. For this, 50 µL of the resazurin suspension was added to each well of the microplate, followed by the addition of 450 µL of DMEM + 10% FBS. Incubation in the dark was carried out for 16 h, after which the plate was read in a spectrophotometer at a wavelength of 570 nm. The optical densities

(ODs) obtained were converted into a percentage of cell viability using the following formula (Equation (1)):

$$\% \text{ Metabolic activity} = (\text{OD treated group} \times 100) / \text{average OD control group} \quad (1)$$

2.10. Statistical Analysis

The obtained data were initially analyzed for normality using D'Agostino, Shapiro–Wilk, and Kolmogorov–Smirnov tests. Data showing normality were evaluated by ANOVA complemented by Turkey's test. Data that did not show normality were evaluated by Kruskal–Wallis complemented by Dunn's test. All analyses utilized a *p*-value of 0.0001.

3. Results

3.1. Antifungal Activity in Planktonic Cultures

The antifungal activity of *J. regia* extract against planktonic cultures of *Candida* species is summarized in Table 1. The MIC was 12.5 mg/mL for *C. dubliniensis*, *C. guilliermondii*, and *C. tropicalis*, indicating higher susceptibility of these species. In contrast, *C. albicans*, *C. glabrata*, and *C. krusei* presented lower sensitivity, with a MIC of 25 mg/mL.

Table 1. Antifungal activity of *Juglans regia* extract in planktonic cultures of *Candida* species.

<i>Candida</i> spp.	MIC	MMC
<i>C. albicans</i>	25 mg/mL	50 mg/mL
<i>C. dubliniensis</i>	12.5 mg/mL	50 mg/mL
<i>C. guilliermondii</i>	12.5 mg/mL	25 mg/mL
<i>C. glabrata</i>	25 mg/mL	25 mg/mL
<i>C. krusei</i>	25 mg/mL	25 mg/mL
<i>C. tropicalis</i>	12.5 mg/mL	25 mg/mL

Legend: MIC—minimum inhibitory concentration; MMC—minimum microbicidal concentration.

The MMC was 25 mg/mL for most species, but higher values were required for *C. albicans* and *C. dubliniensis* (50 mg/mL).

3.2. Anti-Biofilm Action—Biomass

J. regia treatments at 50, 100, and 200 mg/mL reduced the biomass of *C. albicans* biofilms by 31.9%, 32.7%, and 31%, respectively (Figure 1). For *C. tropicalis* and *C. guilliermondii*, biomass reduction increased with concentration, reaching 30.4% and 27%, respectively, at 200 mg/mL, with values comparable or superior to nystatin. *C. krusei* and *C. dubliniensis* showed moderate but consistent reductions (≈ 21 –28%), with the extract outperforming nystatin in the case of *C. dubliniensis*, as shown in Figure 1.

3.3. Anti-Biofilm Action—MTT (Metabolic Activity)

The metabolic activity of *Candida* biofilms was markedly reduced following treatment with *J. regia* extract (Figure 2). The strongest effect was observed against *C. albicans*, with reductions ranging from 61% to 74.3%, which were comparable or superior to nystatin (63.3%). *C. glabrata* also showed a consistent inhibition (≈ 53 –57%), although slightly lower than the reference drug.

For *C. tropicalis* and *C. guilliermondii*, inhibition increased with concentration, reaching 44.9% and 51.3%, respectively, at 200 mg/mL, with the latter surpassing nystatin. In contrast, *C. krusei* and *C. dubliniensis* exhibited only modest responses ($\leq 20\%$ reduction), highlighting species-dependent variability.

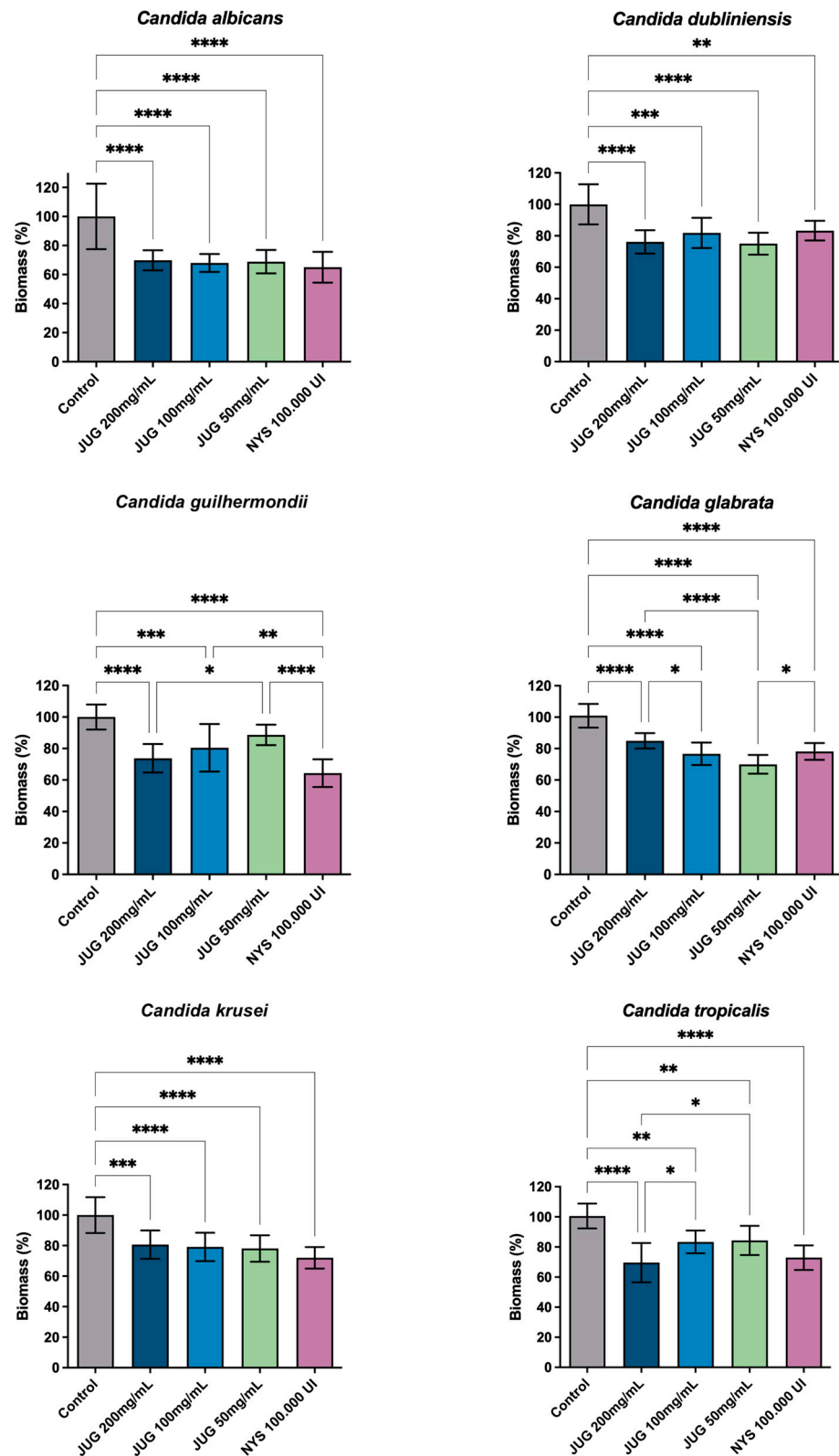


Figure 1. Reduction in the biomass of *Candida* spp. biofilms after treatment for 5 min with *J. regia* extract. Legend: (*) 0.033; (**) 0.002; (***) < 0.0002; (****) < 0.0001; JUG—*Juglans regia* extract; NYS—nystatine.

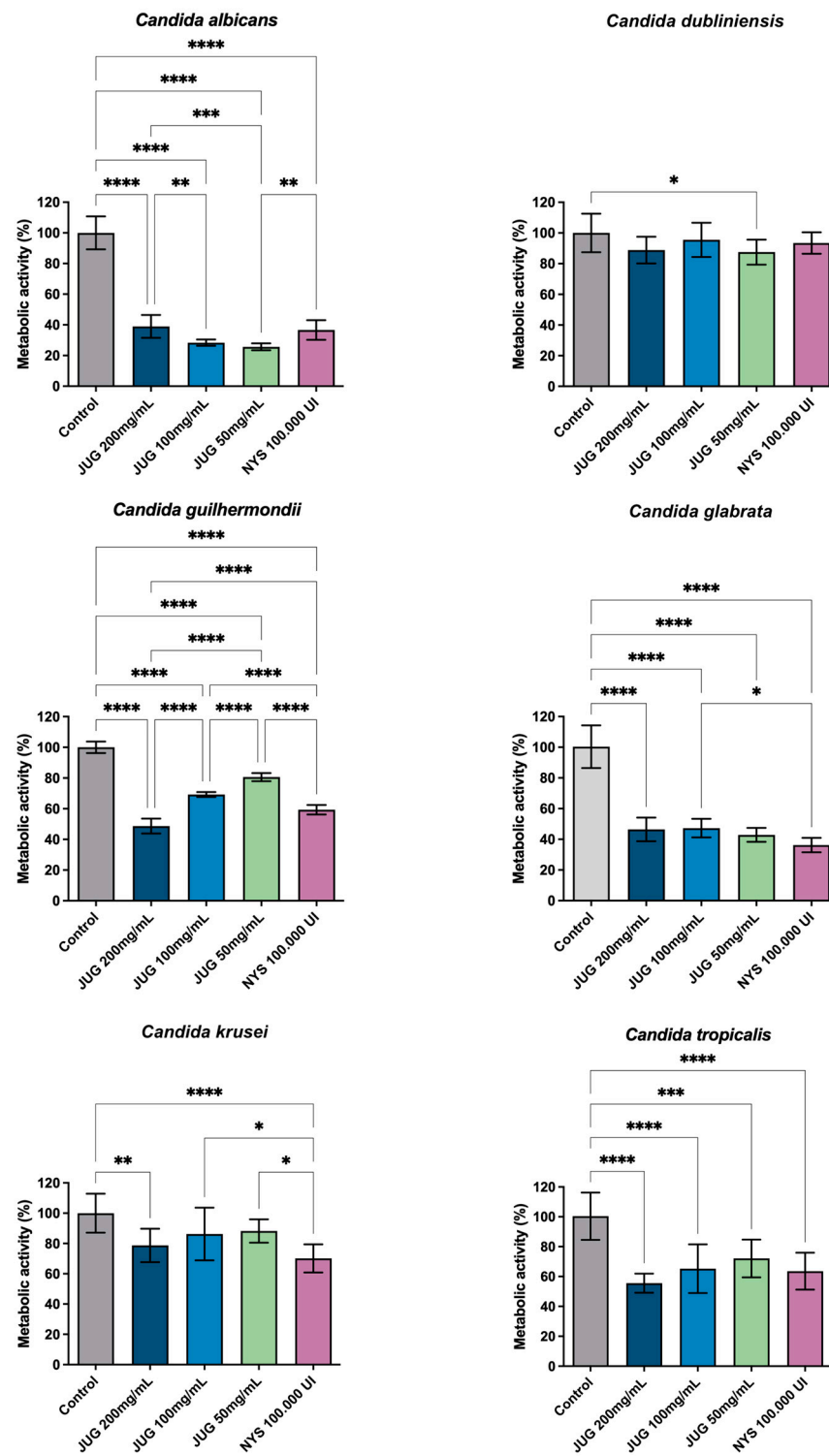


Figure 2. Reduction in the metabolic activity of *Candida* spp. biofilms after treatment for 5 min with *J. regia* extract. Legend: (*) 0.033; (**) 0.002; (***) < 0.0002; (****) < 0.0001; JUG—*Juglans regia* extract; NYS—nystatine.

3.4. Cell Viability by Resazurin Essay

The cytocompatibility of *Juglans regia* extract was assessed on HEPG2 cells using the resazurin assay (Figure 3). The results revealed a non-linear, biphasic dose-response pattern. At intermediate concentrations (25–50 mg/mL), cell viability decreased substantially (≈ 39 –54%), suggesting cytotoxic effects. At higher concentrations

(100–200 mg/mL), however, viability partially recovered (≈ 80 –85%), indicating possible adaptive cellular responses.

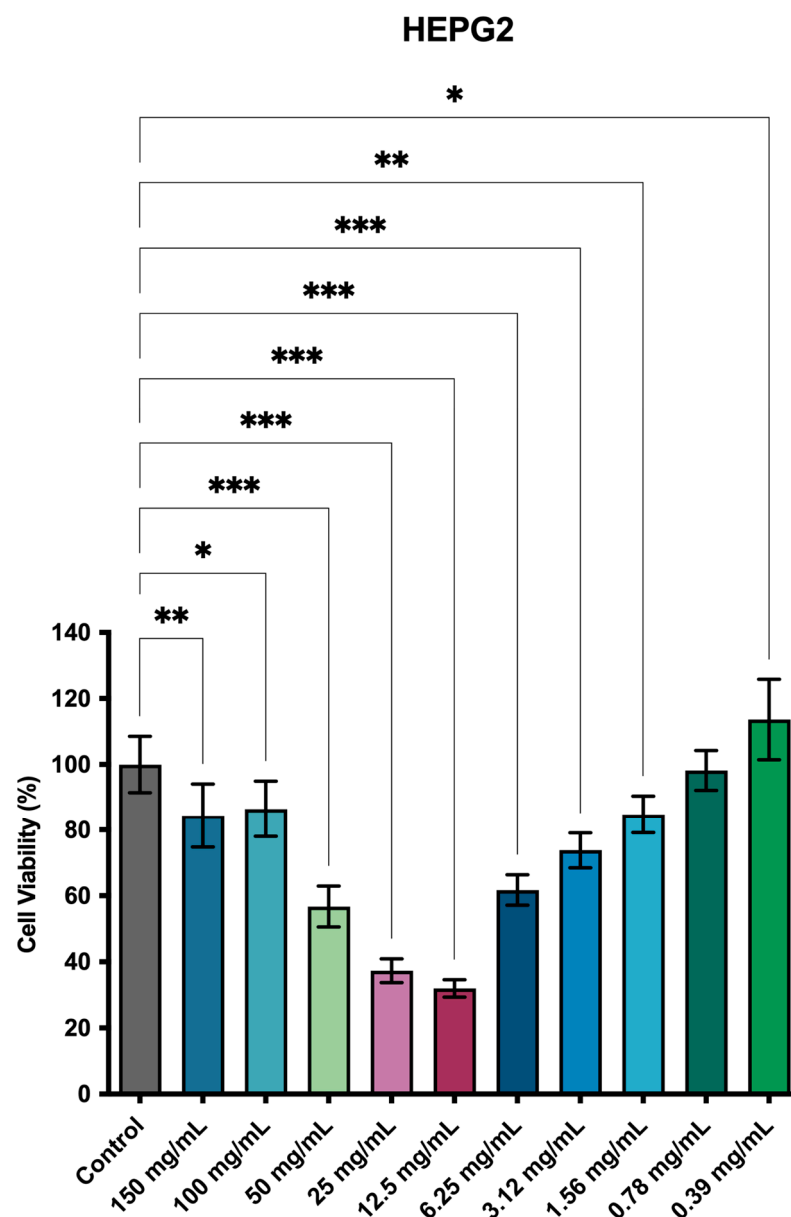


Figure 3. Cytocompatibility of *J. regia* extract applied to human hepatocytes (HEPG2). Legend: (*) 0.033; (**) 0.002; (***) < 0.0002.

At lower concentrations (0.39–3.12 mg/mL), cell viability was maintained or even enhanced ($\geq 100\%$ at 0.39 mg/mL), consistent with a hormetic effect frequently described for plant-derived compounds. This profile suggests that the extract may exert stimulatory effects at very low doses, toxicity at intermediate doses, and relative tolerance at higher exposures.

4. Discussion

The present study investigated the antifungal and cytocompatible properties of a glycolic extract of *Juglans regia* against six clinically relevant *Candida* species. Our findings highlight both the potential and the complexity of using natural extracts in dental applications.

The antifungal activity of *J. regia* in planktonic cultures was species dependent. The MIC and MMC results showed that the antifungal effect of *J. regia* was species-dependent,

with *C. dubliniensis*, *C. guilliermondii*, and *C. tropicalis* exhibiting higher susceptibility, while *C. albicans*, *C. glabrata*, and *C. krusei* required higher concentrations. These results are consistent with prior studies showing variable activity of *J. regia* extracts depending on the solvent used, plant part studied, and fungal strain tested [16–20]. The higher concentrations needed to eradicate *C. albicans* are noteworthy, given its prevalence in oral candidiasis [4]. This suggests that *J. regia* may act in a primarily fungistatic manner against certain species, a finding consistent with those reported by D’Angeli et al. [16] and Noumi et al. [17].

In this study, *J. regia* extract promoted modest reductions in biofilm biomass (≈ 21 – 32%), but a much stronger inhibition of metabolic activity, particularly in *C. albicans* (61 – 74%) and *C. glabrata* ($\approx 54\%$). This indicates that the extract primarily affects cellular metabolism rather than disrupting the biofilm matrix. For instance, *C. glabrata* biofilms exhibited strong metabolic inhibition despite relatively modest biomass reductions, while *C. dubliniensis* showed the opposite profile, with notable biomass reduction but minimal metabolic inhibition. These results suggest that the extract targets distinct aspects of biofilm physiology, depending on the species. Previous reports support this interpretation, as *J. regia* may interfere with key metabolic pathways [23,24], and the sensitivity of each species appears linked to its intrinsic biofilm phenotype [25]. Taken together, these data highlight that *J. regia* extract impacts biofilm viability more strongly than structural mass, an important distinction for interpreting its antifungal potential. These findings reinforce the idea that *J. regia* does not act through a single mechanism, but rather exerts multifactorial effects depending on the fungal species and biofilm structure. This complexity may be advantageous, as multi-targeted agents are less likely to induce resistance compared to single-molecule drugs [1,9].

The cytocompatibility assays, based on OECD guideline 129, revealed a biphasic, hormetic response to *J. regia* extract, which represents one of the most intriguing findings of this study. At low concentrations (0.39 – 3.12 mg/mL), the extract stimulated cell viability, while intermediate concentrations (25 – 50 mg/mL) showed cytotoxic effects, and higher concentrations (100 – 200 mg/mL) partially restored viability. Such U-shaped dose–response curves are well-documented in toxicology and pharmacology, and are particularly common for plant-derived compounds [26]. Hormesis may reflect adaptive responses of mammalian cells, including the activation of antioxidant defense pathways or modulation of stress-response genes. In the case of *J. regia*, this phenomenon could be attributed to the chemical complexity of the extract, which contains diverse metabolites with potentially synergistic or antagonistic interactions. Although preliminary, these findings highlight the importance of conducting a thorough characterization of the bioactive compounds within the extract and elucidating their underlying mechanisms of action [27].

From a translational perspective, this non-linear profile highlights both opportunities and risks. On the one hand, the stimulatory effects at low doses may support the safe use of diluted formulations in preventive products such as mouth rinses. On the other hand, the cytotoxicity observed at intermediate concentrations underscores the importance of dose optimization and careful safety testing before clinical application.

When compared with conventional antifungals, *J. regia* extract showed efficacy comparable to nystatin against *C. albicans* and, in some cases, even superior activity against *C. dubliniensis* and *C. guilliermondii*. However, the extract was less effective against *C. krusei*, a species known for intrinsic resistance to azoles and reduced susceptibility to polyenes [5–7]. This variability emphasizes that *J. regia* is unlikely to replace conventional antifungals, but could serve as a complementary agent. Its potential use in preventive strategies, where complete eradication is not required but suppression of pathogenic overgrowth is beneficial, deserves further investigation [4].

This study presents several limitations. First, the study was conducted exclusively *in vitro*, and results may not translate directly to the oral cavity, where saliva, host immunity, and microbial interactions influence antifungal efficacy. Second, the short exposure time (5 min) was chosen to simulate a mouth rinse, but longer or repeated exposures may be necessary in clinical settings [4]. Third, the extract is chemically complex, and its active components were not directly identified in this study. Previous research suggests that flavonoids such as quercetin, myricetin, rutin, and avicularin contribute to antifungal effects [16,23], but their relative roles remain unclear. Fourth, the cytotoxicity results must be interpreted with caution due to the use of HEPG2 cells, which are immortalized hepatocytes in 2D monolayer configuration. Future studies should include multiple primary cell types relevant to oral application. Finally, a major limitation is that the antifungal concentrations required to inhibit *Candida* spp. (12.5–200 mg/mL) overlap with the concentration range showing cytotoxic effects in HepG2 cells (25–50 mg/mL), which restricts the direct translational potential of the extract.

Despite these limitations, our findings open promising perspectives. The extract could be incorporated into dental materials, varnishes, or mouth rinses designed for patients at risk of oral candidiasis, such as denture wearers, elderly individuals, or immunocompromised patients. Its multifactorial mode of action may reduce the likelihood of resistance development. Future studies should focus on the isolation and characterization of the active compounds, mechanistic studies exploring mitochondrial disruption, oxidative stress induction, or inhibition of efflux pumps, *in vivo* models of oral candidiasis to assess efficacy, bioavailability, and safety and formulation research to optimize delivery and stability in dental products.

5. Conclusions

Thus, the antifungal activity observed not only validates the traditional use of the plant in oral hygiene and health practices, but also suggested its potential as a source of bioactive secondary metabolites for novel antifungal agents. However, the effects were species-dose-dependent, with strong activity against *C. albicans* and *C. glabrata*, but limited action against *C. krusei* and *C. dubliniensis* biofilms. Moreover, given that the concentrations required for antifungal effects are relatively high compared to standard drugs (e.g., nystatin), further work is needed to isolate and characterize the active constituents, optimize formulations, and improve selectivity. Importantly, these results are limited to *in vitro* assays. Further research on the mechanisms of action, toxicity, and *in vivo* efficacy will be essential before considering any translational application. The biphasic profile observed also indicates that caution is required when extrapolating doses, as toxicity may vary depending on the concentration and cell type. While this non-linear response could represent both a challenge and an opportunity for the development of antifungal agents derived from *J. regia*, careful attention to the overlap between antifungal and cytotoxic concentrations will be essential to ensure translational feasibility.

Author Contributions: Conceptualization: D.G.M., L.d.P.R., F.V.M., F.M.T., F.P.R., and F.C.; methodology: D.G.M., L.d.P.R., F.V.M., L.S.N., I.A.F., B.M.G., F.M.T., F.P.R., and F.C.; validation: D.G.M., L.d.P.R., F.V.M., F.M.T., F.P.R., and F.C.; formal analysis: D.G.M., L.d.P.R., F.V.M., F.M.T., F.P.R., and F.C.; investigation: D.G.M., L.d.P.R., F.V.M., L.S.N., I.A.F., B.M.G., F.M.T., F.P.R., and F.C.; resources: D.G.M., L.d.P.R., F.V.M., F.M.T., F.P.R., and F.C.; data curation: D.G.M., L.d.P.R., F.V.M., L.S.N., I.A.F., B.M.G., F.M.T., F.P.R., and F.C.; writing—original draft preparation: D.G.M., L.d.P.R., F.V.M., L.S.N., I.A.F., B.M.G., F.M.T., F.P.R., and F.C.; writing—review and editing: D.G.M., L.d.P.R., F.V.M., F.M.T., F.P.R., and F.C.; visualization: D.G.M., L.d.P.R., F.V.M., F.M.T., F.P.R., and F.C.; supervision: D.G.M., L.d.P.R., F.M.T., and F.C.; project administration: D.G.M., L.d.P.R., F.V.M., F.M.T., F.P.R., and F.C.;

funding acquisition: D.G.M., L.d.P.R., F.V.M., F.M.T., F.P.R., and F.C. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The raw data supporting the conclusions of this article will be made available by the authors upon request.

Conflicts of Interest: The authors declare no conflicts of interest.

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