*Journal of Biomedical Materials Research Part A*

WILEY

Society For Biomaterials

RESEARCH ARTICLE OPEN ACCESS

# Antifungal Activity, Cytocompatibility, and Wound Healing Potential of Novel Mucoadhesive Formulations for Oral Drug Delivery

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**Received:** 7 May 2025 | **Revised:** 15 August 2025 | **Accepted:** 8 September 2025

**Funding:** This work was supported by the Fundação de Amparo à Pesquisa do Estado de São Paulo (2021/15142-1, 2021/14444-4, and 2023/00292-3), the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (001), the Fundação para a Ciência e a Tecnologia (LA/P/0008/2020), and the Conselho Nacional de Desenvolvimento Científico e Tecnológico (313498/2025-3).

**Keywords:** *Candida albicans* | chlorhexidine | cyclodextrins | drug delivery systems | nystatin | oral candidiasis

## ABSTRACT

Conventional treatments for oral candidiasis often fail due to the complexities of the oral environment and the increasing antifungal drug resistance. Therefore, there is a growing demand for new therapies that optimize drug bioavailability, allowing for lower therapeutic doses while enhancing cytocompatibility, maintaining antifungal, anti-inflammatory, and wound healing efficacy. This study investigated the antifungal activity, cytocompatibility, wound healing potential, and mucosal adhesion of novel mucoadhesive formulations containing nystatin (NYS) or chlorhexidine (CHX) complexed with β-cyclodextrin (βCD), compared with the drug-free formulation (GEL) and the standard treatment with 2% miconazole gel (DK—Daktarin). Efficacy against *Candida albicans* was evaluated by measuring the metabolic activity, whereas cytocompatibility with human gingival fibroblasts (HGFs) was analyzed for viability, morphology, lactate dehydrogenase (LDH) release, and apoptosis. Additionally, wound healing potential was investigated by assessing cell migration efficacy, anti-inflammatory activity, and reactive oxygen species (ROS) scavenging activity. Mucoadhesion was evaluated using mucin discs and a texture analyzer. Mucoadhesive gels containing βCD-complexed NYS or CHX exhibited significantly higher antifungal activity when compared to the GEL and DK groups ($p < 0.05$). Compared to fibroblast control cultures, those exposed to drug-complexed gels exhibited similar viability ($p > 0.05$) and morphological parameters, lower LDH release ($p < 0.05$), and similar apoptosis rates ($p > 0.05$). Additionally, exposure to the βCD-modified gels was associated with complete wound closure ($p > 0.05$), significant anti-inflammatory effect, with downregulation of pro-inflammatory gene expression ($p < 0.05$), and higher ROS scavenging activity ($p < 0.05$). The developed formulations showed no difference in mucoadhesiveness ($p > 0.05$), which was superior to that of DK ($p < 0.05$). Therefore, the proposed drug-complexed mucoadhesives are promising therapeutic options for oral candidiasis.

## 1 | Introduction

The management of oral fungal infections, mostly caused by *Candida* species, presents a considerable clinical challenge due to the increasing incidence of antifungal resistance and associated adverse effects [1, 2]. Systemic antifungal therapies require high drug concentrations and often have limited efficacy, particularly at the infection sites, associated with low local bioavailability. In addition, they carry the risk of potential systemic toxicity [3]. As a result, topical management for oral candidiasis has been proposed to address some of these limitations, exploiting its direct access to the infection sites, improving the therapeutic outcomes, and minimizing the adverse effects [2, 4, 5].

Topical formulations of nystatin (NYS), oral suspensions, and miconazole oral gel are the first therapeutic options for oral candidiasis [6, 7]. The mechanism of action of NYS is due to the binding to ergosterol—a sterol—present in the membrane of fungal cells, while that of miconazole is based on the inhibition of the biosynthesis of this component [8]. Although these drugs are effective for mild to moderate infections, clinical studies have reported high recurrence rates 2 weeks after the end of the treatment [6, 7, 9]. Failure of conventional therapy is probably associated with the dynamic environment of the oral cavity and the low mucoadhesivity of these formulations, as the continuous self-cleansing action of saliva, along with constant tongue movement and the swallowing reflex, creates challenges for retaining topical antifungal agents [10]. Furthermore, the anatomical complexity of the oral cavity, with its irregular surfaces, creates niches where *Candida s*pecies can persist, often leading to recurrent infections [11].

In contemporary pharmaceutical formulations, the addition of therapeutic agents into mucoadhesive carriers presents a promising avenue to enhance drug efficacy and patient adherence [5, 12]. Mucoadhesive drug delivery systems present an effective strategy by extending drug residence time at the infection site, enabling sustained drug release and enhancing therapeutic efficacy [5, 13]. Moreover, the incorporation of bioactive agents into mucoadhesive formulations may also reduce the inflammatory responses associated with fungal infections. These pro-inflammatory scenarios contribute to tissue damage and can exacerbate disease severity [5, 13, 14].

Chitosan is a natural, biodegradable, and biocompatible polymer with strong mucoadhesive properties due to its cationic nature, which promotes adhesion to negatively charged mucin in the oral mucosa. Despite its benefits, chitosan's low solubility at physiological pH may limit its penetration-enhancing effect [12]. Hydroxyethyl cellulose (HEC), a water-soluble cellulose derivative, complements chitosan, providing rapid swelling and additional mucoadhesive potential [15, 16]. Together, chitosan and HEC form a hydrogel through hydrogen bonding interactions, creating a biocompatible matrix suitable for drug incorporation [15, 16]. The mechanical and mucoadhesive properties of this hydrogel, including changes due to drug loading, were previously characterized to evaluate its potential as a delivery system for the treatment of oral candidiasis [17].

Among drug delivery system strategies, coupling with beta-cyclodextrin (βCD) has attracted considerable attention due to its unique ability to form inclusion complexes with a wide range of hydrophobic drugs, thereby improving their solubility, stability, and bioavailability [18–20]. Current literature on the cytocompatibility of mucoadhesive formulations containing antifungal agents is still limited, with available studies focusing on preliminary cytotoxicity assays such as MTT [11–25]. These assays, although important as an initial assessment, provide only a superficial view of the safety of the formulations, without considering relevant aspects of cytocompatibility, such as cell morphology, lactate dehydrogenase (LDH) release, and apoptosis. Furthermore, the authors found no information in available published reports on the effects of mucoadhesive formulations on essential biological processes in tissue repair, such as cell migration, anti-inflammatory activity, and control of reactive oxygen species (ROS). These factors are essential for a better understanding of the therapeutic efficacy and safety of mucoadhesive drug delivery systems. This study aims to evaluate the antifungal activity, cytocompatibility, anti-inflammatory and wound healing potential, as well as the mucoadhesive properties of new mucoadhesive formulations containing NYS or CHX complexed with βCD, comparing them with the standard treatment for oral candidiasis with miconazole gel. To the best of the authors' knowledge, this study will be the first to analyze these properties using the conjugation of antifungal drugs with βCD before incorporating the complexes into mucoadhesive formulations. It is expected that understanding the cytocompatibility, cellular response, and immunomodulatory effects of mucoadhesive formulations will contribute to better establish their clinical application.

## 2 | Materials and Methods

### 2.1 | Materials

CHX (99.9% purity) and βCD (> 97% purity) were purchased from Sigma-Aldrich (St. Louis, MO, USA) and NYS (6800.99 IU/mg, CAS 1400-61-9) from Galena Química e Farmacêutica Ltda (Campinas, SP, Brazil). Sabouraud Dextrose Agar (SDA) was purchased from Difco (Detroit, Michigan, USA), and RPMI-1640 medium from Gibco (Thermo Fisher Scientific, Life Technologies Limited, Paisley, UK). MOPS and resazurin sodium salt were purchased from Sigma-Aldrich (St. Louis, MO, USA). Alpha-minimum essential medium (α-MEM) and the following reagents and kits were also purchased from Sigma-Aldrich: MTT bromide, dimethyl sulfoxide (DMSO), paraformaldehyde, Triton-X100, LDH solution (TOX-7), DPPH for antioxidant assays, and *Porphyromonas gingivalis'* lipopolysaccharide (LPS). Fluorescence staining was carried out using Alexa Fluor-488 (Molecular Probes, Eugene, OR, USA) and 4′,6-diamidino-2-phenylindole (DAPI) (BD Biosciences, Franklin Lakes, NJ, USA). The Apoptosis Detection Kit (Annexin V-FITC) was purchased from BioLegend (San Diego, CA, USA). Gene expression analysis was conducted with the CFX384 real-time PCR system and specific primers from Bio-Rad (Hercules, CA, USA). Mucin discs were fabricated using Mucin Type III from Sigma-Aldrich.

### 2.2 | Mucoadhesive Formulations

Briefly, the mucoadhesive gel formulation was prepared by dispersing 2% (w/v) chitosan (Molecular weight: 50,000–190,000;

Da: 75%–85% deacetylated; Sigma-Aldrich, St. Louis, MO, USA) in 1% (v/v) acetic acid aqueous solution by stirring for 120 min at 50°C. Separately, 6% (w/v) hydroxyethyl cellulose (HEC; Natrosol, Ashland Inc., Wilmington, DE, USA) was dispersed in warm distilled water (70°C), and then methylparaben (0.15% w/w), sodium metabisulfite (0.1% w/w), and EDTA (0.1% w/w) were added as preservatives. The chitosan dispersion was then manually mixed with the HEC dispersion in a weight ratio of 1:3 at room temperature until homogenization [17].

Inclusion complexes of NYS with βCD at a 1:1 M ratio and CHX with βCD at a 1:2 M ratio were prepared by dissolving the components in a hydroalcoholic medium (ethanol: water, 1:1 v/v) under continuous stirring at 25°C for 24 h. After removal of the solvent by rotary evaporation at 60°C, the remaining aqueous phase was frozen at −82°C and lyophilized for 72 h [19, 20]. The complexes were characterized by Fourier-transform infrared spectroscopy (FTIR), thermogravimetric analysis (TGA), and differential scanning calorimetry (DSC), as reported in a previous study [17].

Non-complexed or complexed drug powders were incorporated into the mucoadhesive formulations at concentrations determined in previous studies on the characterization of NYS [19] and CHX [20] inclusion complexes with βCD, which demonstrated antifungal activity against all *Candida* strains tested [19, 20]. To ensure consistency and evaluate the effects of the drugs, the formulated mucoadhesive gel was used as a carrier vehicle for NYS or CHX. Additionally, for comparison with the gels developed in this study, the commercially available 2% miconazole gel was included (Daktarin Janssen, Beerse, Belgium), since this formulation is the most widely used as a positive control in clinical trials testing mucoadhesives [9, 25, 26]. Thus, the following study groups were formed:

A. GEL—mucoadhesive without drug (GEL);
B. DK—Daktarin- gel containing 20 mg/g miconazole (DK);
C. NYS 1.61%—mucoadhesive containing 16.1 mg/g NYS (NYS 16.1);
D. NYS 3.2%—mucoadhesive containing 32 mg/g NYS (NYS 32);
E. NYS:βCD—mucoadhesive containing 36 mg/g inclusion complex NYS:βCD, equivalent to 16.1 mg/g NYS (NYS:βCD);
F. CHX 0.48%—mucoadhesive containing 4.8 mg/g CHX (CHX 4.8);
G. CHX 3.2% - mucoadhesive containing 32 mg/g CHX (CHX 32);
H. CHX:βCD - mucoadhesive containing 26 mg/g inclusion complex CHX:βCD, equivalent to 4.8 mg/g CHX; (CHX:βCD).

## 2.3 | Preparation of the Formulations' Leachates

Leachates of the aforementioned mucoadhesive formulations were evaluated for their antifungal activity, cytocompatibility, and wound healing properties. Leachates were prepared in a ratio of 1:3 g/mL in medium at 37°C for 4 h [17, 27]. The leachates were obtained by the spontaneous release in RPMI-1640 medium with MOPS for the microbiological testing and in α-MEM for cell culture assays.

## 2.4 | Characterization of the Formulations' Leachates

### 2.4.1 | Establishment of Cytocompatible Leachate Concentrations

To determine the working concentrations of leachates from formulations for cell cultures, a preliminary screening of a wide concentration range (100% to 1.56%) was performed using L929 mouse fibroblasts (ATCC # CCL-1, Manassas, Virginia, USA), following the ISO Guidelines 10993:5 (2018) [28]. L929 cells were seeded at $10^4$ cell/cm$^2$ and were cultured for 24 h in standard medium (α-MEM containing 10% fetal bovine serum, 100 IU/mL penicillin, and 100 μg/mL streptomycin, all from Sigma-Aldrich), at 37°C and 5% $CO_2$/air. Subsequently, the cultures were exposed to the leachates for 24 h, and cell response was evaluated for viability by the MTT assay (methodology described below). Cytotoxicity was determined as the percentage of cell viability compared to the control group (no leachates, 100% cell viability), and leachates were classified as non-cytotoxic for viability ≥ 70%, considering the cut-off value established by the ISO guidelines [28].

### 2.4.2 | Antifungal Activity

The leachates from the formulations were characterized for antifungal activity using the microdilution method, following the guidelines established by the Clinical Laboratory Standards Institute [29]. A *Candida albicans* strain (ATCC10231) was thawed, plated on a Petri dish with SDA, and incubated at 37°C for 24 h. After growth, distinct colonies were suspended in RPMI-1640, and the cell density was then adjusted to achieve a final concentration of 1–5 × $10^5$ CFU/mL using a spectrophotometer (Synergy Mx Monochromator-Based, Biotek, Winooski, Vermont, USA) set at a wavelength of 530 nm. The assay was performed in 96-well plates, and leachates were added to achieve final concentrations of 50% to 1.56%. The plates were incubated for 24 h, and the antifungal activity was evaluated by measuring the metabolic activity of *C. albicans* by the resazurin assay. Wells without leachates were used as controls. For the assay, 10% v/v of resazurin (0.1 mg/mL, Resazurin sodium salt) was added to each well. Plates were incubated at 37°C for 45 min, and fluorescence was read at 530 nm of excitation and 590 nm of emission using a microplate reader. The results were expressed as relative fluorescence units (RFUs). Assays were performed in triplicate.

### 2.4.3 | Cytocompatibility Assessment in Human Gingival Fibroblasts

A comprehensive analysis of the cytocompatibility of the leachates was performed using HGFs (AG09429, Coriell Cell Repository, Camden, NJ, USA). HGF cultures were exposed to the selected

concentrations determined by the assay in Section 2.4.1. The following leachates were tested: (i) GEL, DK, NYS 16.1, NYS 32, and NYS:βCD, at 25%, 12.5%, and 6.25%, and (ii) GEL, DK, CHX 4.8, CHX 32, and CHX:βCD, at 6.25%, 3.12%, and 1.56%. These concentrations represent the ranges that exhibit selective antifungal toxicity, i.e., targeting *C. albicans* while having minimal impact on the viability of eukaryotic cells (L929 fibroblasts). HGFs, 7th passage, were seeded at a density of $10^4$ cells/cm$^2$ and cultured for 24–72 h to form a cell layer. Subsequently, cultures were exposed to the leachates for 24 h. Cells incubated without leachates were used as controls. This experimental setup was used to evaluate cell viability, morphology, apoptosis, LDH release, and migratory activity as described below.

**2.4.3.1 | Cell Viability.** Adhered HGFs were exposed to leachates from the NYS and CHX formulations, and the cellular viability was evaluated by the MTT assay. A 5 mg/mL solution of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT) was added (10% v/v) per well, followed by incubation at 37°C for 2 h. Supernatants were then discarded, and formazan crystals were dissolved in DMSO. Absorbance was measured at a wavelength of 550 nm using a spectrophotometer (Synergy HT, Biotek Winooski, VT, USA). The assay was performed in quintuplicate.

**2.4.3.2 | Cell Morphology.** After being exposed for 24 h to leachates from the NYS and CHX formulations, HGF cultures were washed with PBS and incubated with a mitochondrial tracking dye (250 nM; MitoSpy Red CMXRos). Subsequently, cells were fixed in 4% paraformaldehyde and permeabilized with 0.1% Triton-X100. Bovine serum albumin at 1% was added, and actin filaments of the cell cytoskeleton (F-actin) were stained with Alexa Fluor-488 (1:100), followed by nuclear staining with DAPI (8 μg/mL). Cells were visualized using the Celena S digital imaging system (Molecular Devices, Sunnyvale, CA, USA). The assay was performed in triplicate.

**2.4.3.3 | Lactate Dehydrogenase Release.** The LDH assay was performed on HGF cultures to evaluate membrane integrity following 24 h exposure to NYS and CHX mucoadhesive leachates. Supernatants were collected and added to the LDH solution of the toxicological assay (TOX-7) for 30 min. The reaction was stopped by the addition of 15 μL/well of HCl (1 M). Absorbance values were obtained by measuring the primary wavelength at 490 nm, with the value at 690 nm subtracted. The assay was performed in triplicate.

**2.4.3.4 | Apoptosis.** HGFs were exposed to the mucoadhesive leachates for 24 h, and apoptosis levels were determined by the Apoptosis Detection Kit (Annexin V-FITC) following the manufacturer's protocol. The fluorescence of stained cells was assessed by flow cytometry (FACScalibur cytometer, BD Biosciences, Franklin Lakes, NJ, USA), and the data were analyzed using FlowJo software (FlowJo v10, Ashland, OR, USA). The assay was performed in triplicate.

### 2.4.4 | Wound Healing Potential

**2.4.4.1 | Scratch Wound Healing Migration.** HGFs were cultured until reaching >70% confluence. Then, the cell monolayer was scratched using a 10 μL micropipette tip, and images of the cell monolayer were immediately captured using an inverted microscope (Zeiss Axiolab 5, Carl Zeiss AG, Aalen, Germany) equipped with a camera (Axiocam 5 Color Camera, Carl Zeiss AG). Subsequently, cells were exposed to NYS and CHX mucoadhesive leachates for 24 h. Following this, images of the cell layer were captured at predetermined time points (24 h and 48 h), and cell migration was analyzed using ImageJ software (version 1.53v; National Institutes of Health, USA). Quantitative assessment was performed in triplicate, considering five random distances.

**2.4.4.2 | Anti-Inflammatory Activity.** The expression of pro-inflammatory genes (*IL1B, IL6, CXCL8*, and *NFKB*) was assessed in LPS-stimulated HGF cultures through quantitative Polymerase Chain Reaction (qPCR) analysis. Briefly, HGF cultures were incubated with 1 μg/mL of *Porphyromonas gingivalis* LPS (Sigma-Aldrich, St. Louis, MO, USA) for 16 h, followed by a 24 h exposure to mucoadhesive leachates containing inclusion complexes. Leachates were assayed at (i) 25% for NYS:βCD, GEL, and DK, and (ii) 3.12% for CHX:βCD, GEL, and DK. Total RNA was extracted from the cell cultures using TRIzol reagent, following the manufacturer's recommendations. RNA concentration and purity were verified by measuring absorbance ratios (A260/A280) with the Take3 module (Gen5, BioTek, Winooski, VT, USA) on a microplate reader (Synergy HT; BioTek). RNA-to-cDNA conversion was performed using a reverse transcription system, and the RNA concentration was set at 250 ng/μL. Quantitative PCR was conducted on a CFX384 real-time PCR system, employing the iTaq Universal SYBR Green Supermix PCR Kit and specific primers, following the manufacturer's cycling protocol. Quality control was verified using Bio-Rad CFX Maestro 1.1 software (v.4.1.24), and only samples with amplification efficiency between 90% and 110% and a linear standard curve ($R^2 > 0.98$) were included in the analysis. The relative quantification of each target gene was normalized to GAPDH as the control gene and calculated using the $2^{-\Delta\Delta Ct}$ method. The assay was performed in triplicate.

**2.4.4.3 | Antioxidant Activity of the Mucoadhesive Gels.** The mucoadhesive gels were tested for ROS scavenging activity by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical reduction assay. Mucoadhesive gels, 15 mg, were dissolved in 435 μL of DPPH ethanol solution to produce a 0.1 mM solution at room temperature under constant shaking. At determined time points (0, 2, and 4 h), the mixture was centrifuged, and aliquots were transferred to a 96-well plate for absorbance measurement at 517 nm using a spectrophotometer. The assay was performed in quintuplicate.

### 2.4.5 | Mucoadhesive Properties

Mucoadhesive properties were evaluated on mucin discs (100 mg mucin, Mucin Type III, Sigma-Aldrich, Cotia, SP, Brazil) using a texture analyzer (Texturometer TX-700, Lamy, France). The formulations (200 mg) were preheated to 37°C and placed in a Petri dish. Mucin discs ($n = 5$) were fixed to a 36 mm aluminum probe with double-sided tape and moistened with 0.1 mL of artificial saliva. The probe moved at 1 mm/s with 0.5 N compression for 120 s, then retracted until detachment. Force-distance graphs

were generated to determine adhesiveness (N), the work of adhesion (N.s), which represents the energy required for detachment, and the debonding distance (mm). Specimens were made in duplicate.

### 2.4.6 | Statistical Analysis

Data are presented as mean ± standard deviations and were analyzed using appropriate statistical tests, with normality assessed by the Shapiro–Wilk test ($p > 0.05$). Statistical significance was determined using one-way or two-way ANOVA, followed by post hoc tests ($p < 0.05$), specifically Dunnett's multiple comparisons tests. Analyses were performed using JAMOVI software (version 2.2, R version 4.0, Sydney, Australia) and GraphPad Prism (version 9.2.0283, Boston, MA, USA).

## 3 | Results and Discussion

This study proposes a novel strategy to address oral fungal infections through the development of mucoadhesive formulations incorporating NYS, a polyene macrolide, and an antimicrobial bisbiguanide (CHX) in their non-complexed and complexed forms with βCD. These two drugs were selected because, when incorporated into a polymeric matrix (soft liners), they demonstrated effectiveness in inhibiting *C. albicans* biofilm at lower minimum inhibitory concentrations (MICs) that were eightfold lower for NYS and fourfold lower for CHX compared with miconazole [30]. Furthermore, they result in no detrimental effects on the fundamental physical and mechanical properties of the carrier polymers, which were not observed with miconazole under most experimental conditions [31–34].

The mucoadhesive gels offer several advantages for treating oral candidiasis, including prolonged retention in the oral cavity, improved drug delivery, and enhanced patient compliance due to their ease of application and reduced dosing frequency [5]. The incorporation of βCD increases the solubility and stability of these antifungal agents, allowing for effective therapeutic concentrations at lower doses [17, 19, 20, 25].

Representative macroscopic images of each formulation are shown in Figure 1, illustrating the visual appearance of the mucoadhesive gels for all study groups. The GEL (A, unloaded formulation) appeared translucent and homogeneous, serving as a visual control. The commercial reference formulation, Daktarin (B), exhibited a whitish, opaque, and homogeneous appearance. Gels containing non-complexed NYS (C and D) showed increased turbidity and yellowish coloration concentration, suggesting limited solubility. In contrast, the complexed NYS formulation (E) was more uniform and slightly opaque, indicating better dispersion. CHX-loaded gels without βCD (F and G) showed heterogeneous appearances with visible undissolved particles or agglomerates, especially at higher concentrations, evidencing low solubilization. CHX:βCDl (H), however, was clearer and more homogeneous, reinforcing the benefit of complexation with cyclodextrin.

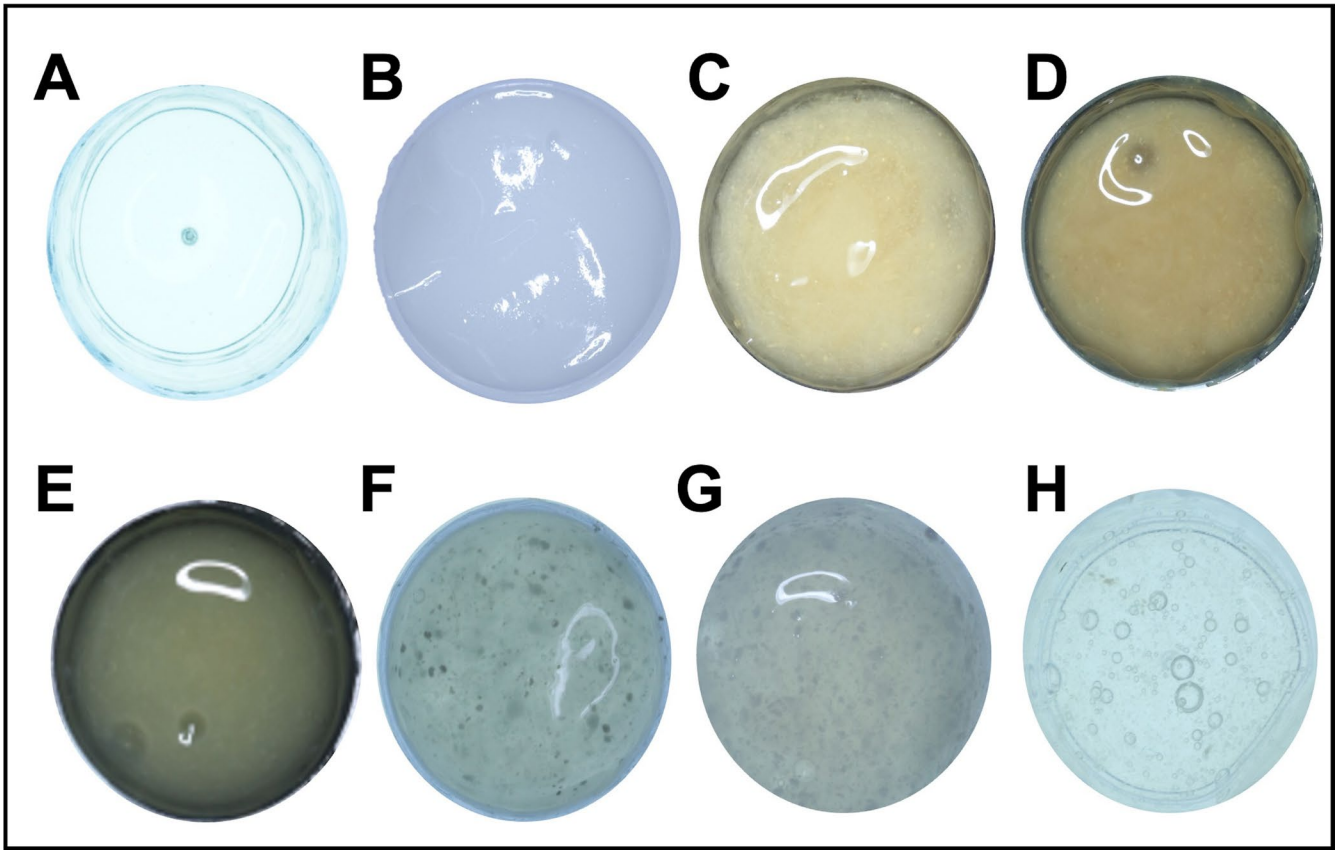


**FIGURE 1** | Representative macroscopic images of the mucoadhesive formulations used in this study. Visual appearance of the gels from the following groups: (A) GEL (vehicle control), (B) DK, (C) NYS 16.1, (D) NYS 32, (E) NYS:βCD, (F) CHX 0.48, (G) CHX 32, and (H) CHX:βCD.

**TABLE 1** | Mean values of percentages ±standard deviations (%) of cell viability/proliferation (MTT assay) of L929 murine fibroblasts after 24 h of exposure to mucoadhesive leachates of all prepared formulations ($n = 3$) at concentrations ranging from 100% to 1.56%.

| Groups | 100% | 50% | 25% | 12.5% | 6.25% | 3.12% | 1.56% |
|---|---|---|---|---|---|---|---|
| Gel | 53 ± 9[a] | 100 ± 12 | 100 ± 17 | 85 ± 19 | 76 ± 18 | 88 ± 3 | 84 ± 12 |
| DK | N/A | N/A | N/A | 49 ± 14[a] | 60 ± 7[a] | 64 ± 16 | 90 ± 9 |
| NYS 16.1 | 43 ± 6[a] | 44 ± 24[a] | 100 ± 5 | 100 ± 15 | 100 ± 10 | 100 ± 18 | 100 ± 12 |
| NYS 32 | 32 ± 8[a] | 24 ± 6[a] | 66 ± 16 | 100 ± 3 | 100 ± 12 | 100 ± 20 | 100 ± 8 |
| NYS:βCD | 75 ± 2 | 94 ± 5 | 96 ± 9 | 100 ± 15 | 100 ± 5 | 100 ± 13 | 100 ± 9 |
| CHX 4.8 | N/A | N/A | N/A | 25 ± 8[a] | 59 ± 6[a] | 57 ± 7[a] | 72 ± 4 |
| CHX 32 | N/A | N/A | N/A | 25 ± 9[a] | 43 ± 23[a] | 63 ± 11 | 65 ± 12 |
| CHX:βCD | N/A | N/A | N/A | 25 ± 3[a] | 21 ± 12[a] | 72 ± 17 | 70 ± 12 |

*Note:* Data were normalized by the control group (no leachate) set as 100% (0.324 ± 0.07 AU). N/A, not assessed due to high toxicity observed at lower concentrations. Assays were performed in quintuplicate.
[a]Significant differences from control ($p < 0.0001$; 1-way ANOVA, Dunnett's multiple comparisons test).

### 3.1 | Establishment of Leachate's Cytocompatible Concentrations

In a screening assay, leachates from the mucoadhesive formulations were tested for cytotoxicity on L929 mouse fibroblasts over a concentration range of 100% to 1.56%. After 24 h of exposure, cellular viability was assessed (Table 1, with additional information provided in the Supporting Information). The GEL formulation exhibited minimal toxicity, with only the undiluted leachates exhibiting an inhibition of about 50%. DK was lethal at concentrations up to 25% and caused dose-dependent inhibitory effects at lower levels (12.5% to 1.56%), which corroborates the findings of Lam et al. [35], who reported that miconazole triggers ROS accumulation, leading to keratinocyte cytotoxicity and potentially impairing wound healing. Non-complexed NYS formulations caused dose-dependent inhibition at levels 100% to 25%, but they were harmless at lower concentrations. However, it is important to highlight that the complexed NYS formulation (NYS:βCD) showed no deleterious effects throughout the concentration range tested. Even the cultures exposed to the undiluted leachates maintained a viability of approximately 75%, exceeding the cytotoxicity cut-off value (70%) defined by ISO guidelines 10,993 (2018) [28]. On the other hand, CHX formulations exhibited higher toxicity, being lethal up to 25% and presenting concentration-dependent inhibitory effects at levels ≤12.5%, with cellular viability being around 70% in the lower concentration range (6.25% to 1.56%). These results are consistent with the findings of Moraes et al. [36], who evaluated the survival rate (%) of *Artemia salina* after exposure to NYS, NYS:βCD, CHX, CHX:βCD, or βCD. Their study demonstrated that both non-complexed and βCD-complexed NYS exhibited low cytotoxicity. In contrast, CHX exhibited a dose-dependent cytotoxic profile, and similarly, complexation with βCD did not significantly alter the toxicity of CHX, further supporting the observations made in the present investigation.

### 3.2 | Antifungal Activity of the Mucoadhesive Leachates

The leachates were assessed for their antifungal activity against *C. albicans* at different concentrations. Table 2 presents the results of metabolic activity (RFUs) for the most representative concentrations of the experiment (additional information provided in the Supporting Information). The GEL and DK leachates did not show antifungal effects or statistical differences compared to the control ($p > 0.05$). The absence of antifungal activity of DK corroborates the low efficacy of miconazole against *Candida* spp. found in previous in vitro [30] and in vivo [6] studies. Regardless of the concentration tested, all groups of non-complexed or complexed drugs (NYS or CHX) significantly decreased the metabolic activity of *C. albicans* compared to the control ($p < 0.05$). NYS formulations exhibited strong antifungal activity, with complete inhibition observed at the tested levels (NYS 16.1, NYS 32, and NYS:βCD).

In the CHX groups, CHX 4.8 showed strong antifungal activity at concentrations up to 6.25%, while CHX 32 exhibited almost complete inhibition at concentrations up to 1.56%. Importantly, complexed CHX:βCD showed a significant reduction in fungal metabolic activity at lower concentrations (3.12% and 1.56%) compared with that observed with the mucoadhesive containing the same amount of non-complexed drug (CHX 4.8 mg/g).

Notably, formulations containing CHX:βCD showed antifungal activity at reduced CHX concentrations (more than threefold) compared with those of the free drug carriers. These findings agree with a recent in vivo study, which showed that soft relining materials containing non-complexed or βCD-complexed antifungal agents promoted tissue recovery in *Candida*-associated denture stomatitis, with the complexed formulations proving effective at considerably lower concentrations than the non-complexed drugs [37].

### 3.3 | Cytocompatibility of the Mucoadhesive Leachates

Based on the previous findings concerning the cytotoxicity screening and the antifungal activity of mucoadhesive leachates (Sections 3.1 and 3.2), their cytocompatibility was detailed within specific concentration ranges, namely 25%–6.25% for the NYS

**TABLE 2** | Mean values ±standard deviations (RFUs) of metabolic activity of *C. albicans* ATCC 10321 after 24 h of exposure to mucoadhesive leachates from all prepared formulations ($n = 3$) at different concentrations.

| Groups | 12.5% | 6.25% | 3.12% | 1.56% |
|---|---|---|---|---|
| Gel | 23,283.15 ± 1277.03 | 19,213.15 ± 1814.08 | 17,608.65 ± 1875.95 | 15,766.49 ± 2469.34 |
| DK | 12,612.49 ± 2290.02 | 13,383.15 ± 1071.87 | 14,238.15 ± 1872.42 | 10,364.15 ± 2097.86 |
| NYS 16.1 | 145.49 ± 74.14[a] | 218.82 ± 113.01[a] | 257.49 ± 70.06[a] | 245.49 ± 18.5[a] |
| NYS 32 | 129.49 ± 77.78[a] | 113.49 ± 112.86[a] | 195.82 ± 43.73[a] | 181.49 ± 95.7[a] |
| NYS:βCD | 312.49 ± 112.25[a] | 383.82 ± 45.65[a] | 407.15 ± 54.00[a] | 387.15 ± 42.00[a] |
| CHX 4.8 | 144.82 ± 66.91[a] | 189.49 ± 43.15[a] | 9844.65 ± 2478.41[b] | 10,240.82 ± 2939.8[b] |
| CHX 32 | 88.49 ± 59.60[a] | 143.82 ± 50.05[a] | 139.15 ± 53.00[a] | 158.82 ± 28.68[a] |
| CHX:βCD | 83.15 ± 37.51[a] | 144.82 ± 8.02[a] | 145.49 ± 3.51[a] | 2819.49 ± 1650.96 [a,b] |
| CONTROL | 16,283.82 ± 2692.31 | | | |

*Note:* Assays were performed in triplicate.
Abbreviation: RFUs, relative fluorescence units.
[a]Significant differences from the control.
[b]Significant differences across varying concentrations within the same experimental group ($p < 0.0001$; 1-way ANOVA, Dunnett's multiple comparisons test).

formulations (NYS 16.1, NYS 32, and NYS:βCD) and 6.25%–1.56% for the CHX preparations (CHX 4.8, CHX 32, and CHX:βCD).

Cytocompatibility was assessed in HGFs due to their relevance in damaged mucosal surfaces associated with *C. albicans* overgrowth and tissue invasion. These cells contribute to local immune responses, regulate inflammation, and serve as critical mediators in host-pathogen interactions, particularly regarding the ability of *C. albicans* to invade connective tissues [38]. Cytocompatibility was evaluated by metabolic activity, cell morphology, LDH release, and apoptosis rate of HGFs after a 24-hour exposure to leachates.

As shown in Figure 2A, HGF cultures incubated with the leachates from the GEL and NYS groups reached metabolic activity values close to 100%, with no statistically significant differences compared to the control. For the CHX groups, the decrease in metabolic activity was observed only with CHX:βCD leachates at 6.25%. DK leachates exhibited inhibitory effects at the highest concentrations tested (i.e., 25%, 12.5%, and 6.25%) (Figure 2A,B).

Figure 2C,D show fluorescence images of HGFs representing their morphology, F-actin cytoskeleton organization, and mitochondria distribution, after exposure to leachates at concentrations of 25% for NYS groups or 3.12% for CHX groups. Prominent nuclei, well-organized F-actin fibers, numerous cytoplasmic extensions, and intense perinuclear mitochondria could be observed in control cells. In addition, HGFs exposed to the plain mucoadhesive gel leachate (GEL group) or containing NYS at low concentrations (NYS 16.1 and NYS:βCD) exhibited an appearance indistinguishable from the control. On the other hand, morphological changes were noted in the NYS 32 group, with reduced cytoplasmic volume. The DK group demonstrated a reduced number of cells throughout the evaluation time with decreased cell-to-cell contacts, particularly evident in 25% (Figure 2C) and noticeable in 6.25% (Figure 2D). For the CHX groups, only HGFs incubated with CHX 32 showed morphological changes with reduced cytoplasmic volume (Figure 2D). Cytoskeleton integrity is crucial to maintain cell homeostasis, as it supports intracellular transport and influences protein distribution. Therefore, alterations in the cytoskeletal structure may reflect disturbed cellular processes, such as impaired migration or response to stimuli, as observed in the mucoadhesive groups containing high concentrations of non-complexed antifungals (NYS 32 and CHX 32) [39]. Moreover, mitochondria (in red) were positioned close to the nucleus, an indicator of cellular health. Mitochondrial structure and function play an important role in cellular biology, contributing to calcium homeostasis, ROS generation, and apoptosis regulation [40, 41].

LDH levels released by HGF cells after exposure to mucoadhesive leachates are shown in Figure 3. As expected, cells treated with Triton-X (positive control) showed high values of LDH release, confirming membrane damage. Compared to the negative control, exposure to DK at 25% caused a slight increase in LDH release, while significantly lower values were observed after incubation with leachates from all experimental groups (GEL, NYS, and CHX). This low toxicity is consistent with the results on cellular metabolic activity.

External perturbations can trigger apoptosis, a tightly regulated energy-dependent process involving caspase activation and chromosome fragmentation [40]. To assess this, flow cytometry was conducted to quantify the proportions of live, early apoptotic, late apoptotic, and necrotic HGF cells after 24 h of exposure to mucoadhesives' leachates (25% in the NYS group and 3.12% in the CHX group). Results are presented in Tables 3 and 4, with additional information provided in the Supporting Information. The apoptotic effects of the leachates containing NYS (NYS 16.1, NYS 32, and NYS:βCD) or CHX (CHX 4.8, CHX 32, and CHX:βCD) were insignificant and comparable to those observed in the GEL group ($p > 0.05$).

These findings indicate that the interaction of HGFs with leachates from the mucoadhesive gels proposed in this study did not affect the metabolic activity, cell membrane integrity, or apoptotic

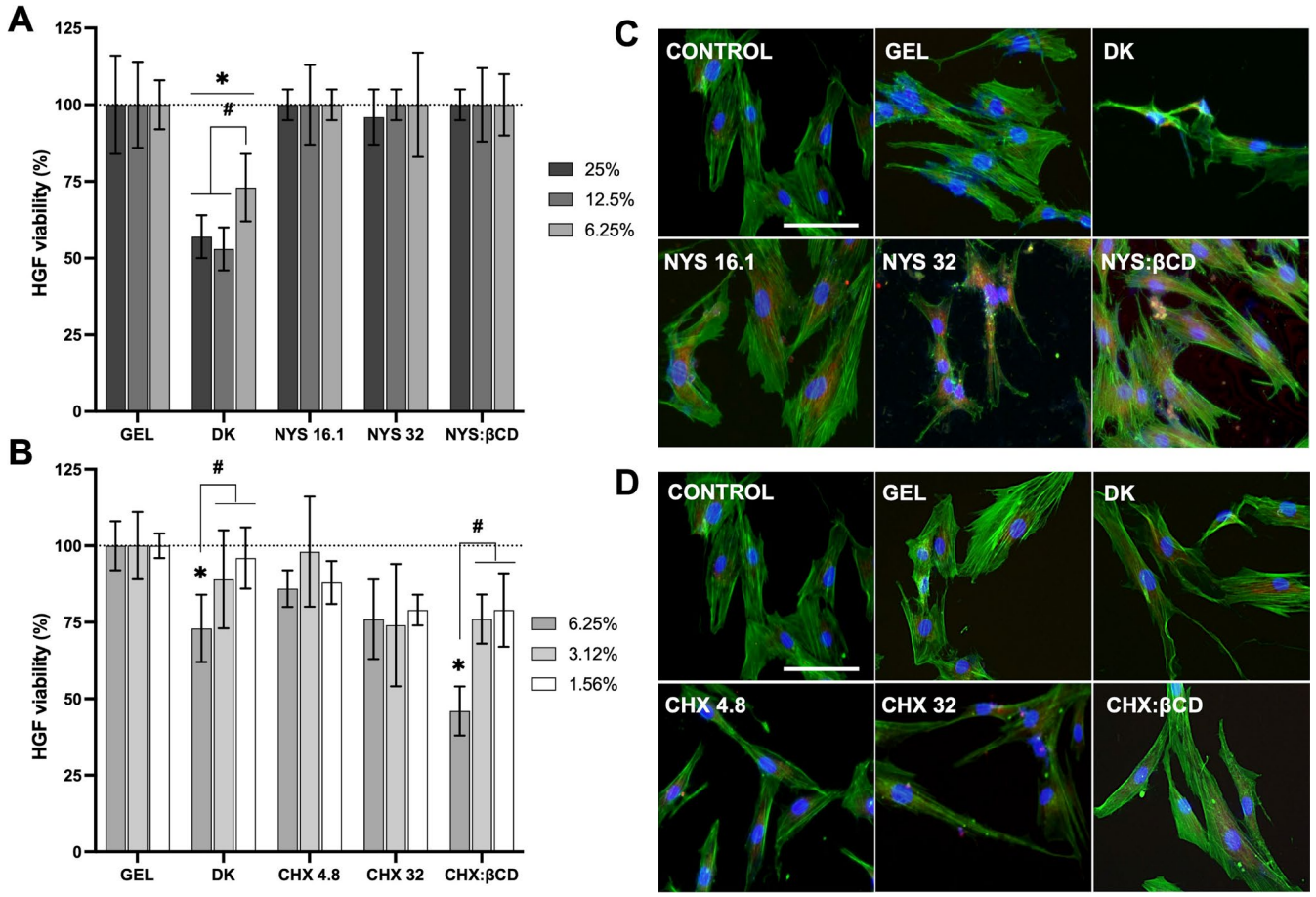


**FIGURE 2** | Cytocompatibility of leachates from mucoadhesive formulations in HGF cultures ($n = 5$). (A, NYS and B, CHX) Cellular metabolic activity (%) after 24 h exposure. Error bars represent standard deviations. Assays were performed in quintuplicate. Data were normalized by the control group (no leachates) set as 100% (0.195 ± 0.014 AU). #Significant differences between experimental groups. *Significant differences from control ($p < 0.0001$; 1-way ANOVA, Dunnett's multiple comparisons test). (C, NYS and D, CHX) Representative fluorescence images of cultures stained for F-actin (green), mitochondria (red), and nucleus (blue) ($n = 3$) exposed for 24 h to mucoadhesive leachates at concentrations of 25% (NYS groups) or 3.12% (CHX groups). Scale bar 100 μm.

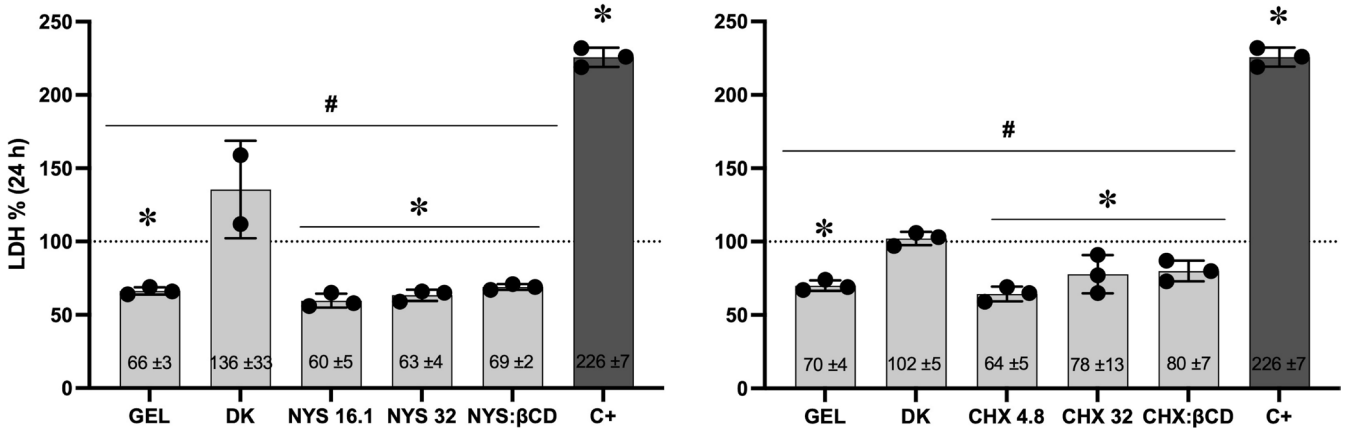


**FIGURE 3** | Percentages (%) of lactate dehydrogenase (LDH) released by HGF cells exposed for 24 h to mucoadhesive leachates ($n = 3$) from (A) GEL, DK, NYS 16.1, NYS 32, and NYS:βCD groups at 25%, or (B) GEL, DK, CHX 4.8, CHX 32, and CHX:βCD groups at 3.12%. Error bars represent standard deviations. Assays were performed in triplicate. Data were normalized to the control (no leachates) set at 100% (0.176 ± 0.004 AU; measured at 490–690 nm; dotted line). The positive control (C+) consisted of cells treated with the LDH assay lysis solution (L2152, Triton-X), which induces cell membrane lysis. *Significantly different from the control (no leachates) ($p < 0.01$). #Significantly different from the positive control (C+) ($p < 0.0001$); 1-way ANOVA, Dunnett's multiple comparisons test.

**TABLE 3** | Mean values ±standard deviations (%) of the apoptotic effect of mucoadhesive leachates ($n = 3$) from the GEL, DK, NYS 16.1, NYS 32, and NYS:βCD groups at 25% in HGFs after 24 h of exposure.

| | GEL | DK | NYS 16.1 | NYS 32 | NYS:ßCD |
|---|---|---|---|---|---|
| Live cells | 93.5 ± 3.0 | 95.7 ± 3.1 | 94.0 ± 3.42 | 91.7 ± 5.6 | 95.9 ± 2.3 |
| Early apoptosis | 1.4 ± 0.5 | 1.0 ± 0.5 | 1.9 ± 1.1 | 2.3 ± 1.3 | 0.3 ± 0.1 |
| Late apoptosis | 4.2 ± 2.5 | 3.1 ± 2.6 | 3.8 ± 2.2 | 5.4 ± 3.9 | 2.5 ± 2.0 |
| Necrosis | 0.9 ± 0.1 | 0.2 ± 0.1 | 0.3 ± 0.1 | 0.7 ± 0.4 | 1.2 ± 0.2 |

*Note:* Results are expressed as percentages of labeled apoptotic cells marked by Annexin V/PI staining. No statistically significant differences were observed between groups ($p > 0.9999$; 1-way ANOVA, Dunnett's multiple comparisons test). Assays were performed in triplicate.

**TABLE 4** | Mean values ±standard deviations (%) of the apoptotic effect of mucoadhesive leachates ($n = 3$) from the GEL, DK, CHX 4.8, CHX 32, and CHX:βCD groups at 3.12% in HGFs after 24 h of exposure.

| | GEL | DK | CHX 4.8 | CHX 32 | CHX:ßCD |
|---|---|---|---|---|---|
| Live cells | 88.2 ± 4.8 | 88.8 ± 4.4 | 92.2 ± 5.1 | 92.1 ± 5.4 | 86.2 ± 10.1 |
| Early apoptosis | 1.7 ± 0.3 | 3.2 ± 0.8 | 0.7 ± 0.3 | 1.6 ± 1.1 | 1.2 ± 0.7 |
| Late apoptosis | 8.7 ± 4.5 | 7.7 ± 3.6 | 6.1 ± 4.5 | 6.0 ± 4.7 | 10.8 ± 8.4 |
| Necrosis | 1.1 ± 0.5 | 0.2. ±0.1 | 0.9 ± 0.2 | 0.4 ± 0.1 | 2.0 ± 0.6 |

*Note:* Results are expressed as percentages of labeled apoptotic cells marked by Annexin V/PI staining. No statistically significant differences were observed between groups ($p > 0.9999$; 1-way ANOVA, Dunnett's multiple comparisons test). Assays were performed in triplicate.

rate. The high cytocompatibility of NYS-containing mucoadhesives, even at high concentrations, was expected. Being a polyene, NYS binds to ergosterol, a key component of the fungal cytoplasmic membrane, resulting in alterations in its cellular permeability [42]. Ergosterol is a specific component of the cell membrane in fungi and some protozoa, playing a role similar to cholesterol in humans. In contrast, CHX is a cationic compound that binds nonspecifically to negatively charged phospholipids in the cell membrane [43]. This lack of specificity may be responsible for the cytotoxicity observed with CHX formulations in mammalian cells [44–46]. In this study, however, the proposed CHX formulations allowed for the identification of a concentration range that demonstrated both antifungal activity and cytocompatibility with HGFs.

The cytocompatibility results of this study on miconazole gel agree with those of Lam et al. [35], who demonstrated that miconazole induces ROS accumulation, leading to cytotoxicity in keratinocytes and potentially impairing wound healing. Additionally, the lower performance of the DK leachates in the cells could be attributed to their excipients, which include ethylene glycol derivatives and butylated hydroxyanisole. Although these compounds are considered safe for human use, in vitro experimental conditions demonstrated cytotoxic effects due to increased cationic influx that can lead to ROS generation and glutathione reduction [47]. βCD complexation of NYS (NYS:βCD) increased its cytocompatibility, as supported by Moraes et al. [36], who found that NYS:βCD was less toxic to *A. salina* than its non-complexed form. Although the complexation allowed the use of much lower concentrations of CHX, optimizing its antifungal efficacy, its cytotoxic effects were not attenuated as initially expected.

The nanostructure of the βCD complexes is known to protect and increase the release rate of drugs from their matrix, in addition to controlling cellular interactions [48]. In fact, as pointed out by Garcia et al. [17], drug-βCD complexes presented 1.5 to 4 times higher release rates when compared to non-complexed drugs, increasing their bioavailability. As a result, these formulations allow the stability and optimization of the antimicrobial activity of drugs within a controlled release system, ultimately contributing to better therapeutic outcomes [49–55]. This is especially advantageous to poorly soluble agents, such as NYS and CHX, as it also minimizes potential adverse pharmacological effects. The formation of βCD complexes alters the behavior of the drug, improving cytocompatibility with host cells [56] and enhancing antifungal activity [57]. This is consistent with the findings of the present study, in which complexation with βCD reduced the cytotoxicity of NYS formulations (Table 1) and increased the antifungal activity of CHX preparations (Table 2). This greater effectiveness can be attributed to the chemical affinity of βCD molecules for cell membranes through hydrogen bonds. As demonstrated by Teixeira et al. [57], the association of CHX with βCD led to drastic changes in the structure of the fungal membrane, such as permeabilization, leakage of ergosterol, and intracellular substances.

### 3.4 | Healing Properties of the Mucoadhesives

The results described in the previous sections demonstrated that the NYS and CHX mucoadhesive formulations exhibited antifungal activity within a concentration range that also maintained clear cytocompatibility with mammalian cells. The potential healing properties of these formulations were analyzed in HGF cultures by the wound closure assay and the expression of pro-inflammatory genes. Additionally, the ROS scavenging activity of the mucoadhesive gels was evaluated by the DPPH assay.

The wound scratch assay was performed in HGF cultures exposed for 24 h to leachates from the formulations containing NYS (25%)

and CHX (3.25%). The migratory activity of the HGFs was assessed over a period of 48 h, and the results are shown in Figure 4 (additional information provided in the Supporting Information). The scratch was successfully created in all groups, producing a clear gap between cell segments (0 h). After 24 h of incubation with the leachates, cell migration was observed across all groups. However, wound closure in the DK group was statistically lower in comparison to the other experimental groups and the control ($p < 0.05$). After 48 h, complete wound closure was observed in all groups, except for the DK group ($p < 0.05$).

No previous studies have evaluated the migratory capacity of fibroblasts exposed to NYS. Regarding CHX, fibroblasts lost their wound closure ability at a concentration of 0.002% after 3 min of contact [45]. At 0.1%, the wound closure rate after 48 h was only 9.67% [58]. However, in the present study, leachates from the proposed mucoadhesive gels exhibited no deleterious effects on the migration capacity of HGF cultures. In fact, cultures incubated with the NYS and CHX leachates tended to show faster wound closure, as demonstrated by the results observed at 24 h. Such results were expected, since the previous cell viability assays revealed no substantial differences compared to the control groups at these leachate concentrations. Notably, cultures exposed to the leachates from DK, a commercially available formulation, were unable to completely close the wound in this in vitro test.

LPS-stimulated HGF cultures were exposed to the leachates from mucoadhesive formulations containing the complexed NYS (NYS:βCD, 25%) or CHX (CHX:βCD, 3.12%) and were evaluated for the levels of pro-inflammatory genes (i.e., *IL1B, IL6, CXCL8*, and *NFKB*) (Figure 5). The incubation of HGFs with LPS resulted in a significant increase in the expression of all assessed genes ($p < 0.05$). Exposure to leachates from the GEL and NYS:βCD groups showed a clear reduction in the expression of the evaluated genes ($p > 0.05$). Moreover, incubation with CHX:βCD leachates resulted in a downregulation in the expression of most inflammatory genes ($p > 0.05$), except for *IL1B*, compared to the LPS-stimulated cells ($p < 0.05$). No statistical differences were found between the experimental groups. Regarding the DK group, no significant reduction in the expression of these genes was observed after exposure to leachate when compared to LPS-stimulated cultures ($p < 0.05$). A similar trend was also observed in in vivo scenarios, corroborating the absence of intrinsic anti-inflammatory properties of the formulation [59].

NYS is not traditionally recognized for direct anti-inflammatory effects. Oppositely, CHX has intrinsic anti-inflammatory properties, associated with the inhibition of pro-inflammatory cytokines such as IL6 and IL8 in fibroblasts and macrophages exposed to bacteria [60]. However, in the present study, no significant differences in anti-inflammatory activity were found between the GEL and CHX:βCD groups, indicating that the main effects were generated by the carrier itself. Despite this, complexation with βCD may provide controlled and sustained NYS/CHX release, potentially reducing inflammation associated with fungal infections. It has been proposed that βCD can enhance the efficacy of controlled-release formulations and contribute to decreasing the expression of inflammatory genes [61].

Additionally, chitosan, the base compound of the gel tested in this investigation, exhibits anti-inflammatory effects by suppressing

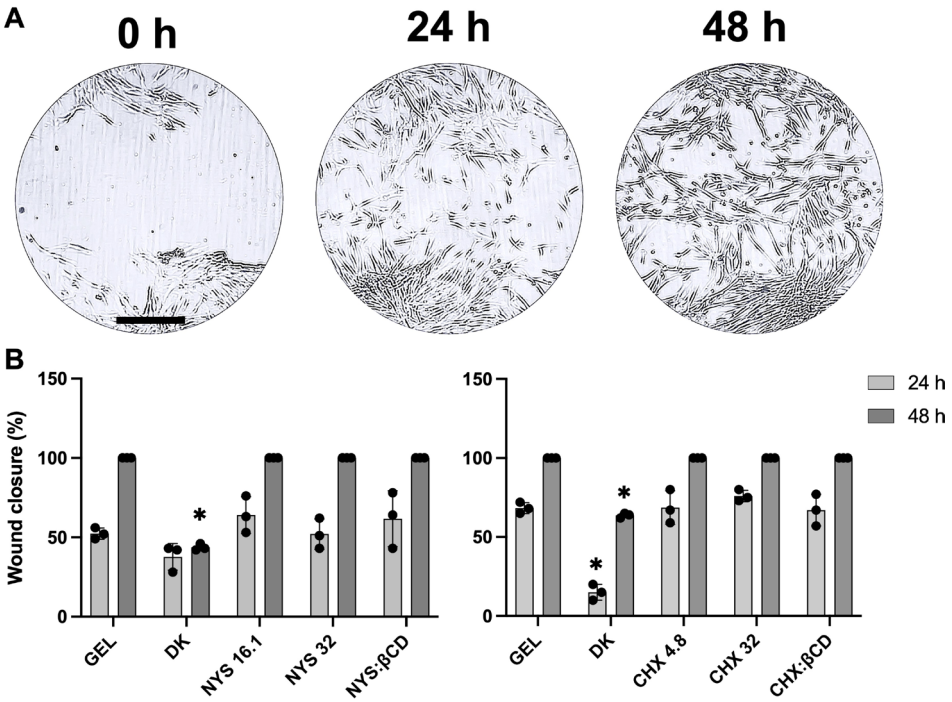


**FIGURE 4** | In vitro scratch assay in HGF cultures exposed to 25% (NYS) or 3.12% (CHX) at 24 h and 48 h ($n = 3$). Error bars represent standard deviations. (A) Representative images of the scratch technique, illustrating the wound closure. Scale bar 100 μm. (B) Quantitative assessment was performed in triplicate, and wound width was measured in μm, considering five random distances in each sample: Results expressed as a percentage of wound closure (%) in relation to the initial width (126.886 ± 10.330 μm). *Significantly different from the control (no leachates) ($p < 0.0001$; 1-way ANOVA, Dunnett's multiple comparisons test).

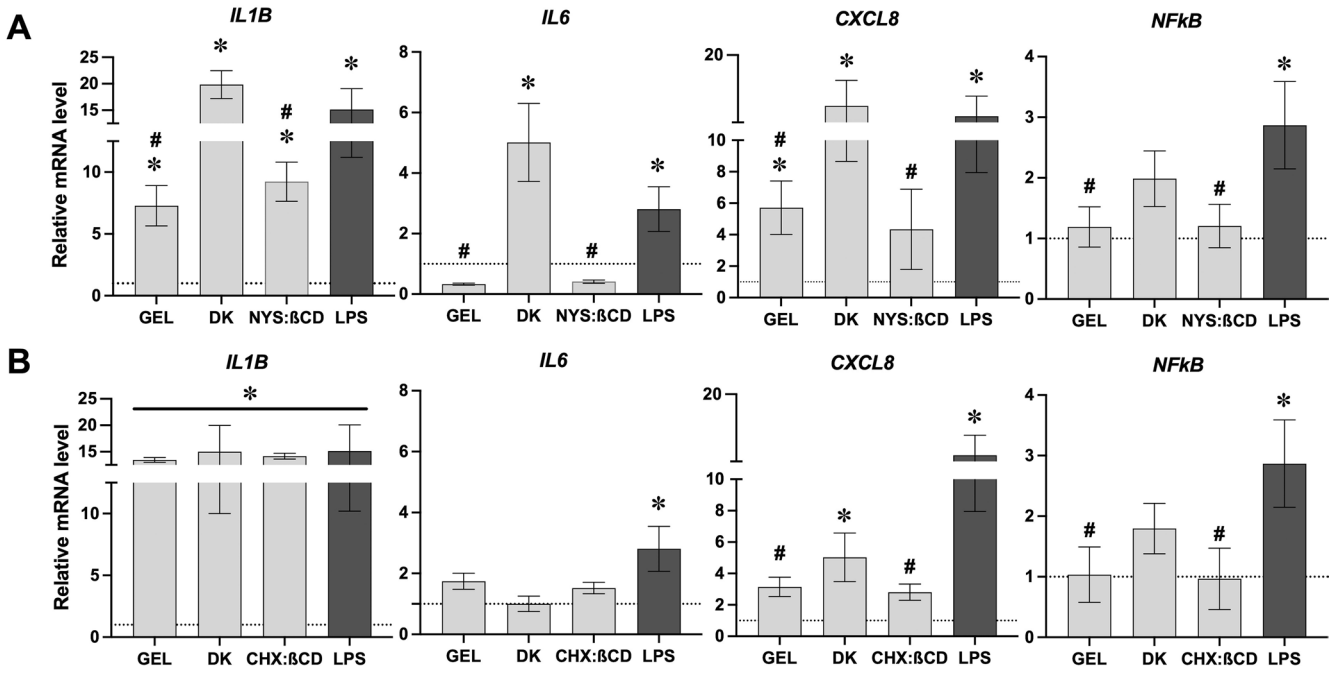


**FIGURE 5** | Gene expression of pro-inflammatory genes by LPS-stimulated HGFs exposed to the mucoadhesive leachates ($n = 3$). Error bars represent standard deviations. Assays were performed in triplicate. (A) NYS:βCD, GEL, and DK extracts at 25%; (B) CHX:βCD, GEL, and DK extracts at 3.12%. Values were normalized by the housekeeping gene (*GAPDH*) and the gene expression levels of control cultures using the $2^{-\Delta\Delta Ct}$ method, represented by the dashed line. *Significantly different from the control (no leachates). #Significantly different from the positive control (LPS-stimulated cultures) ($p < 0.01$; 1-way ANOVA, Dunnett's multiple comparisons test).

the expression of pro-inflammatory cytokines like NF-κB, TNF-α, IL-6, and IL-1β, as well as by preventing the upregulation of Bcl2, IL-1β, and IL-8 by modulating NF-κB pathways [62]. One specific mechanism involves the interaction of chitosan with Toll-like receptors (TLRs) on immune cells such as macrophages. TLRs play a crucial role in the recognition of pathogen-associated molecular patterns (PAMPs), which induce the production of pro-inflammatory cytokines. Chitosan disrupts intracellular signaling pathways activated by TLRs, which alters the cytokine profile released by immune cells, resulting in a decreased inflammatory response [63]. On the other hand, the DK formulation does not contain anti-inflammatory compounds.

The ROS scavenging activity of the mucoadhesive gels was evaluated by the DPPH assay, and the results are shown in Table 5. A significant reduction in the percentage of stable ROS was observed between 0 and 2 h in the GEL, NYS:βCD, CHX 4.8, and CHX:βCD groups ($p < 0.05$). Between 0 and 4 h, significant decreased values were noted in the GEL, NYS 16.1, NYS:βCD, and CHX:βCD groups ($p < 0.05$). Most of the ROS neutralization occurred in the first 2 h, suggesting a rapid scavenging activity. No ROS reduction was detected in DK or CHX 32 groups, indicating that DK formulation could not neutralize ROS from the medium. Moreover, higher concentrations of CHX in the formulation may impair the ROS scavenging properties of the mucoadhesive.

The mucoadhesive gel proposed in the present study is composed of chitosan, which demonstrated antioxidant properties [64–66]. Chitosan monomers have amino and hydroxyl groups that can interact with free radicals, providing scavenging capabilities. The results indicate that the antioxidant activity of the gel was maintained after 2 and 4 h of evaluation ($p < 0.05$), even when complexed βCD drugs were incorporated. Furthermore, evidence suggests that βCD can increase the stability of extracted antioxidants [67].

**TABLE 5** | Mean values of percentages ±standard deviations (%) of the radical scavenging activity of the mucoadhesive gels in the DPPH assay ($n = 5$) at selected time points.

| Groups | 0 h | 2 h | 4 h |
|---|---|---|---|
| GEL | 100% ± 7.87% | 72% ± 2.82%[a] | 65% ± 1.84%[a] |
| DK | 100% ± 9.85% | 100% ± 26.29% | 100% ± 6.44% |
| NYS 16.1 | 100% ± 2.95% | 88% ± 4.70% | 73% ± 0.92%[a] |
| NYS 32 | 88% ± 12.80% | 77% ± 5.63% | 69% ± 2.76% |
| NYS:βCD | 100% ± 5.90% | 73% ± 7.51%[a] | 70% ± 0.92%[a] |
| CHX 4.8 | 100% ± 9.85% | 74% ± 1.56%[a] | 79% ± 14.73% |
| CHX 32 | 100% ± 0.98% | 100% ±15.96% | 100% ± 36.95% |
| CHX:βCD | 100% ± 8.86% | 70% ± 1.39%[a] | 73% ± 0.92%[a] |

*Note:* Results are expressed as a percentage of fluorescence intensity (FI %) normalized to the control (no leachates) (0 h = 0.168 ± 0.009; 2 h = 0.176 ± 0.004; 4 h = 0.180 ± 0.003). Assays were performed in quintuplicate.
[a]Significantly different compared to 0 h in the same experimental group ($p < 0.002$; 1-way ANOVA, Dunnett's multiple comparisons test).

## 3.5 | Mucoadhesive Properties

The results of the mucoadhesive properties of the formulations from different study groups are presented in Table 6. Regarding adhesiveness, no statistically significant differences were

**TABLE 6** | Mean values ± standard deviations of the mucoadhesive properties ($n = 5$) of the formulations.

| Groups | Adhesiveness (N) | Work of adhesion (N.s) | Debonding distance (mm) |
|---|---|---|---|
| GEL | 2.95 ± 0.09 A | 2.68 ± 0.41 BC | 4.78 ± 0.86 AB |
| DK | 1.68 ± 0.18 A | 1.37 ± 0.53 D | 3.31 ± 0.75 B |
| NYS 16.1 | 3.37 ± 0.87 A | 3.97 ± 0.28 A | 6.42 ± 0.73 A |
| NYS 32 | 3.25 ± 0.13 A | 2.98 ± 0.23 AB | 4.13 ± 0.46 AB |
| NYS:βCD | 3.89 ± 0.18 A | 3.44 ± 0.22 AB | 4.01 ± 0.14 AB |
| CHX 4.8 | 2.90 ± 0.03 A | 2.99 ± 0.13 AB | 4.69 ± 1.20 AB |
| CHX 32 | 3.02 ± 0.79 A | 1.65 ± 0.18 CD | 2.19 ± 0.16 B |
| CHX:βCD | 3.73 ± 0.51 A | 3.67 ± 0.05 AB | 4.00 ± 0.13 AB |

*Note:* Different letters indicate statistically significant differences between groups. ANOVA 1-way, Dunnett's multiple comparison test (adhesiveness: $p = 0.09$; work of adhesion: $p = 0.0002$; debonding distance: $p = 0.0078$). Specimens were made in duplicate.

observed between the groups when compared to the control gel (GEL group), indicating that the incorporation of the complexed and non-complexed drug resulted in no change in the mucoadhesive properties of the gel. Adhesiveness is described as the maximum force required to detach the sample from the mucosal surface. It reflects the instantaneous strength of the mucoadhesive bond, influenced primarily by molecular interactions such as hydrogen bonds, van der Waals forces, and electrostatic interactions.

The parameters work of adhesion (N.s) and debonding distance (mm) show variable results; however, there was no difference when comparing the GEL group and the groups with the drug formulations complexed with βCD. This result indicates a homogeneous dispersion of the inclusion complex in the gel, without affecting its mucoadhesive properties. The work of adhesion corresponds to the area under the force–distance curve recorded during the debonding phase of the texturometric analysis. It represents the total energy required to overcome the adhesive interactions, thus providing a comprehensive assessment of mucoadhesive performance. Unlike adhesiveness alone, this parameter captures both the magnitude and persistence of the adhesive force. The debonding distance refers to the distance at which the adhesive force persists as the probe is withdrawn until complete separation occurs, determining the adhesion persistence property of the material [68, 69].

For work of adhesion, four formulations differed significantly from the GEL group. DK and CHX 32 groups exhibited significantly lower values ($p < 0.05$), indicating reduced mucoadhesive performance. In contrast, NYS 16.1 and CHX:βCD groups showed significantly higher work of adhesion ($p < 0.05$), with CHX:βCD standing out among all CHX-based formulations, reinforcing the positive impact of βCD on mucoadhesion. Regarding the debonding distance, the CHX 32 group was the only formulation to exhibit a significantly lower value compared to the GEL group ($p < 0.05$), indicating impaired mucoadhesive retention at higher drug concentrations when uncomplexed. In contrast, NYS:βCD and CHX:βCD groups maintained debonding distances comparable to the control, despite the presence of active compounds. Notably, the NYS:βCD group showed values similar to the NYS 16.1 group, the highest among all groups, while the CHX:βCD group restored the distance lost in the non-complexed CHX 32 group.

Mucoadhesive characteristics directly impact drug bioavailability by ensuring prolonged contact with oral tissues and facilitating the application of the formulations, thus increasing therapeutic efficacy [70]. Indeed, reduced mucoadhesion in DK reduces its retention time on the mucosal surface, which in turn compromises its efficacy, which may be associated with the relatively low cure rate of this drug for oral candidiasis in clinical settings [6, 9].

In this study, the enhanced mucoadhesive properties can be primarily attributed to the chitosan-HEC carrier, as cellulose-based polymers are known for their bioadhesive nature. These findings align with a study conducted on ibuprofen-βCD inclusion complexes in gels [71], which revealed that mucoadhesion was mainly determined by the formulation itself rather than the βCD. Conversely, as observed in the ROS scavenging capacity, the incorporation of higher concentrations of pure antifungal drugs, as observed in the CHX 32 group, resulted in a notable change in mucoadhesive properties.

## 4 | Conclusions

Based on the findings of this study, the novel mucoadhesive formulations containing βCD-NYS or CHX complexes demonstrated in vitro mucoadhesiveness, antifungal activity, cytocompatibility, anti-inflammatory, and healing potential. Additionally, NYS:βCD leachates exhibited improved cytocompatibility, while CHX:βCD demonstrated enhanced antifungal activity when compared to those of non-complexed carriers at similar concentrations.

The results highlight the potential of the proposed formulations as a local therapeutic alternative for oral candidiasis, demonstrating their efficacy and tolerability. To advance toward clinical application, in vivo *studies* are needed to validate the clinical efficacy, long-term safety, appropriate dosage, and application protocols of these formulations in patients with oral candidiasis.

## Conflicts of Interest

The authors declare no conflicts of interest.

## Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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## Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Data S1:** Supporting Information.