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In Vitro Antimicrobial and Antibiofilm Efficacy of an Aminochalcone-Loaded Hydrogel Against *Candida* spp.

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Abstract

Background: Prosthetic candidiasis remains a significant clinical challenge, particularly due to the ability of *Candida* species to form resilient biofilms on dental prostheses, which limits the efficacy of conventional antifungal treatments. In this context, developing strategies to prevent or reduce biofilm formation is essential. Objectives This study investigates the antifungal and antibiofilm potential of a hydrogel formulation incorporating aminochalcone AM-35 as a candidate for the prevention and treatment of prosthetic candidiasis. Methods: To achieve this, experiments were conducted to determine the minimum inhibitory concentration (MIC) of aminochalcone AM-35 against *Candida albicans* and *Candida tropicalis* strains. AM-35 was incorporated into a hydrogel, which was subsequently tested on biofilms formed by these yeast species, both individually and in combination. The experimental disks were sterilized and incubated with *C. albicans*, *C. tropicalis*, and a mixture of both strains for 120 h to allow biofilm maturation. After contamination, the samples were divided into four experimental groups: Group 1: Hydrogel; Group 2: Hydrogel+AM-35; Group 3: Sodium hypochlorite (positive control); and Group 4: No treatment. The samples were then subjected to a sonication process to disaggregate the cells, which were then cultured on plates for colony-forming unit (CFU/mL) counts. The hydrogel's toxicity was evaluated in vivo using the *Galleria mellonella* model. Results: The hydrogel formulation demonstrated significant antimicrobial activity, with an MIC of 7.8 µg/mL for *C. albicans* and 3.9 µg/mL for *C. tropicalis*. Treatment with the hydrogel at a concentration of 39 µg/mL resulted in a significant reduction in the formation and viability of mixed-species biofilms ($p < 0.05$). Additionally, the results indicated robust activity against *C. albicans* and *C. tropicalis* without presenting toxicity in the *Galleria mellonella* model. In conclusion, the hydrogel formulation exhibited effective antibiofilm activity, significantly reducing the microbial load. Conclusions: These findings open new possibilities for the development of alternative treatments for prosthetic candidiasis. The research suggests that the use of chalcone-based compounds may represent a promising approach in combating fungal infections in dentistry.

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Keywords: aminochalcone; biofilm; candidiasis; toxicity; *Galleria mellonella*

1. Introduction

Infections caused by *Candida* species in the oral cavity are common, particularly among individuals who wear dental prostheses. Denture stomatitis is a frequent condition characterized by inflammation of the mucosa in contact with the prosthesis, affecting between 20% and 80% of denture wearers worldwide [1]. Contributing factors include poor oral and prosthesis hygiene, prolonged use—especially overnight—and the presence of mature polymicrobial biofilms, predominantly composed of *Candida* species [2]. Similar to oral biofilms, denture biofilms tend to harbor *Candida* and respiratory pathogens. Among the various *Candida* species, *C. albicans* is the most strongly associated with the development of denture stomatitis [3,4]. These microorganisms exhibit a high affinity for polymethylmethacrylate (PMMA) surfaces, particularly those with surface roughness, facilitating the formation of resilient biofilms that are difficult to eradicate with conventional disinfectants [1,2]. Therefore, proper oral and prosthesis hygiene is essential, as the removal of these biofilms presents a significant clinical challenge [5].

Both *Candida albicans* and *Candida tropicalis* form highly resistant biofilms that significantly complicate clinical treatment. The biofilm formed by *C. albicans* is characterized by a heterogeneous structure composed of yeast cells, pseudohyphae, and hyphae embedded in an extracellular matrix rich in β-1,3-glucan, which acts as a physical and chemical barrier to antifungal penetration. Furthermore, this biofilm is regulated by a complex transcriptional network that controls adhesion, filamentation, and maturation [6,7]. *C. tropicalis*, in turn, produces biofilms consisting of yeast cells, pseudohyphae, and hyphae with intense filamentous budding, associated with high expression of efflux pumps (such as MDR1), which confer resistance to azole antifungals and worsen clinical prognosis. Invasive infections caused by *C. tropicalis* in the biofilm state also significantly increase mortality rates, especially in patients with candidemia, and are associated with poorer clinical outcomes [7,8].

Despite the wide availability of antifungal agents, prosthetic stomatitis (PS) frequently recurs following pharmacological therapy, often requiring repeated treatment interventions. The recurrence of infection is strongly associated with the persistence of Candida biofilms adhered to both the oral mucosa and the inert surfaces of dental prostheses. These biofilms exhibit a complex and highly organized structure, providing protection to fungal cells against the action of antifungal agents and the host's immune mechanisms. This intrinsic resistance of biofilms poses a significant challenge to the complete eradication of the infection and is considered one of the main factors contributing to therapeutic failure and the chronic nature of the condition [9].

Currently, several chemical disinfectants are commercially available for denture hygiene, typically formulated for immersion protocols. Some products combine chemical and mechanical cleaning actions through effervescence. The active ingredients commonly include hypochlorites, alkaline peroxides, enzymes, acids, or EDTA, specifically designed for use with prosthetic materials [2,10]. However, sodium hypochlorite-based solutions can cause corrosion of metal components in dentures. Even in full PMMA prostheses, prolonged exposure to hypochlorites may lead to surface degradation and discoloration. Alternative methods, such as microwave disinfection, have been proposed [11]; however, their long-term effects have not been thoroughly evaluated in clinical settings [12]. Despite its proven efficacy, microwave disinfection may induce boiling of the surrounding water, potentially leading to deformation of the denture base and compromising its functionality,

making it unsuitable for routine patient use [13]. Consequently, there is a pressing need to develop safer and more effective hygiene strategies.

In this context, the development of innovative disinfection approaches is warranted. One promising alternative involves the use of bioactive hydrogels. Hydrogels are three-dimensional polymeric networks that can be employed alone or as carriers for therapeutic agents [14]. Due to their biocompatibility, versatility, and tunable properties—including controlled release of bioactive compounds—they have demonstrated considerable therapeutic potential in oral applications [15].

Chalcones are aromatic ketones belonging to the open-chain flavonoid family, naturally present in various fruits, vegetables, teas, and medicinal plants. These compounds exhibit a wide range of biological activities, including anti-inflammatory, antimicrobial, antioxidant, antidiabetic, and neuroprotective effects [16]. Notably, chalcones have shown synergistic antifungal activity against *C. albicans* [17]. Furthermore, recent studies indicate that chemical modification of chalcones, such as the addition of amino groups, enhances their antifungal efficacy [18].

Therefore, the aim of this study was to evaluate the antimicrobial efficacy of a hydrogel functionalized with aminochalcone, with potential application in the treatment of denture stomatitis.

2. Material and Methods

2.1. Preparation of AM-35

Chalcones were synthesized by Claisen–Schmidt aldol condensation, as reported by Garcia and collaborators (2021) [19]. Reactions were prepared at room temperature using 5.0 mmol of respective benzaldehyde derivatives and 5.0 mmol of respective aminoacetophenones and dissolved in ethanol (50 mL). A catalyst solution of sodium hydroxide in ethanol (1 mol L^{-1}) was added to the reaction medium. Reagent conversion was monitored by thin-layer chromatography. Crude products were poured onto ice from distilled and deionized water and filtered. All compounds were purified over a silica gel chromatography column eluted with hexane and ethyl acetate in a 8:2 ratio.

2.2. Preparation of Hydrogel (H+AM35)

To obtain the hydrogel, an adaptation of the procedure described by Gratieri et al. (2010) [20] was carried out. For this, 1% chitosan was dispersed in a 1% acetic acid solution that was under magnetic stirring (1000 rpm) for 24 h. After this period, the dispersion was placed in an ice bath, and later 16% (*w*/*v*) of poloxamer 407 was added. Subsequently, the dispersion was under magnetic stirring for a period of 50 min until obtaining a hydrogel with a homogeneous aspect and a transparent aspect. For incorporation of the asset (AM-35), it was crushed in porcelain grains with the aid of the pestle and later the hydrogel was added, making a manual mixing for approximately five minutes.

2.3. Preparation of Specimens

Circular wax matrices measuring 40 mm in diameter by 4 mm in height were prepared. These wax matrices were then included in plastic microwave flasks with type III stone plaster in a ratio of 100 g of powder to 30 mL of water. After the plaster's crystallization, the flasks were pressed in a bench hydraulic press for 1 h to prevent any misalignment. The flasks were then opened, immersed in warm water for approximately 3 min to remove the wax matrices, and subsequently cleaned with a solution of household detergent and warm water to remove any traces of wax and Vaseline. Next, the plaster molds were coated with a layer of laboratory silicone, and a resin disc measuring 20 mm in diameter by 2 mm in height was pressed onto the silicone mold to form the sample mold. The inclusion plaster surfaces

were isolated with sodium alginate-based insulation before pressing the acrylic resin. Acrylic resin specimens were made using a volumetric polymer/monomer ratio of 21 g of polymer to 7 mL of monomer, following the manufacturer's recommendation. The resin was manipulated until it reached the plastic phase, inserted into the silicone molds, covered with dampened cellophane, and pressed in a bench hydraulic press. The resin pressing process involved a slow and gradual initial press to remove excess material until reaching 850 kgf for 5 min, followed by a final press of 950 kgf for 30 min. The acrylic resin was polymerized in a household microwave oven according to the manufacturer's recommended cycle. After cooling, the specimens were deflasked, and any rough irregularities were removed using a low-speed bur. The finishing was performed with abrasive stones, silicon carbide sandpaper, and a water-cooled polisher. Samples were immersed in water and stored in an oven at 37 °C [7].

2.4. Microorganism Strains and Culture Conditions

Candida tropicalis ATCC 750 and *Candida albicans* ATCC MYA 2876 strains were used in the study. Microorganisms were cultured separately in medium Sabouraud Dextrose Broth (SDB) (Difco®, Detroit, MI, USA) for 18 h at 35 °C, respectively. *Candida albicans* and *C. tropicalis* were cultured aerobically.

2.5. Determination of Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC)

The Minimum Inhibitory Concentration (MIC) of the hydrogel containing AM-35 was determined against Candida tropicalis ATCC 750 and Candida albicans MYA 2876, following the protocol M27-A2 of the Clinical and Laboratory Standards Institute (CLSI, 2008) [21]. The hydrogel containing AM-35 (H+AM35) was diluted in culture medium and tested at concentrations ranging from 62.5 µg/mL to 0.48 µg/mL. Sodium hypochlorite was used as the positive control for both pathogens. The Minimum Fungicidal Concentration (MFC) was determined by subculturing 10 µL aliquots from each concentration onto Sabouraud Dextrose Agar (SDA) plates (Difco®, Detroit, MI, USA), followed by incubation at 35 °C for 24 h. The MFC was defined as the lowest concentration at which no visible fungal growth was observed on the SDA medium.

2.6. Method for Time-Kill Assay

For the assay, test tubes containing 2.5×10^3 fungal cells per mL in 9 mL of culture medium were used. To these tubes, 1 mL of the formulation containing H+AM35 at a concentration of 1×MFC was added. Subsequently, 300 µL aliquots were transferred to microtubes. The culture medium used for yeast cells was RPMI-1640 (Sigma, St. Louis, MO, USA). The microtubes were incubated at 37 °C. At 0, 2, 4, 6, 10, 12, 18, and 24 h, 30 µL aliquots were collected and seeded onto the surface of BHI agar plates using a sterile Drigalski. After 24 h of incubation, the plates were counted to determine the number of colony-forming units (CFU) per mL. The values were transformed into $\log_{10}$ and plotted on graphs for visualization. All experiments were performed in triplicate [22].

2.7. Effects of Hydrogel Containing AM-35 in Mono- and Mixed Biofilms of C. tropicalis and C. albicans in Acrylic Resin Specimens

Biofilms were formed on acrylic resin discs in 24-well plates using a disc holder, as described by Bombarda et al. (2019) [23]. The resin discs were sterilized under ultraviolet light for 30 min. The artificial saliva used was proposed by Bombarda et al., 2019 [23] (2 g yeast extract (Oxoid, Basingstoke, United Kingdom), 5 g peptone protease (Sigma-AldrichSigma, St. Louis, MO, USA), 2.5 g partially purified type III mucin (Sigma-AldrichSigma, St. Louis, MO, USA), 0.35 g NaCl (Sigma-AldrichSigma,

St. Louis, MO, USA), 0.2 g $CaCl_2$ (Sigma-AldrichSigma, St. Louis, MO, USA), and 0.2 g potassium chloride (Sigma-AldrichSigma, St. Louis, MO, USA). All components were autoclaved at 121 °C for 15 min, except for the mucin, which was filtered and then added to the rest. For biofilm formation, a volume of 1 mL of artificial saliva containing *C. tropicalis* or *C. albicans* cells (1×10^7 CFU/mL) was added to wells containing the acrylic resin disc. For biofilm formation of two species, 500 µL of each suspension (2×10^7 CFU/mL) was used. The plates were statically incubated in aerobic conditions at 35 °C for 24 h to promote cell adhesion on the discs. After this period, each disc was washed with 500 µL saline to remove remnants of dead and non-adherent cells. The H+AM35 was diluted to obtain a concentration of 5X the MIC value, and the biofilms were treated once daily for 1 min for a period of 120 h with 24 h intervals. A total of 1% sodium hypochlorite [24] was used as a positive control, and culture medium (artificial saliva) was used as a negative control. All assays were performed independently and in triplicate [25].

2.8. Methodology for Evaluating Acute Systemic Toxicity in Galleria mellonella

This assay was conducted to evaluate the acute toxic effects of a hydrogel containing AM-35, as described by Megaw et al., 2015 [26]. Fifteen larvae per group, each weighing between 0.2 and 0.3 g and showing no signs of melanization, were used. Ten microliters of pure hydrogel, hydrogel containing AM-35, or 0.9% saline were injected into the hemocoel of each larva through the last left proleg using a Hamilton syringe (Hamilton Company, Reno, NV, USA). The larvae were incubated in the dark, and survival was recorded at selected intervals over 72 h. Larvae that did not move upon touch and exhibited high levels of melanization were counted as dead [26].

2.9. Statistical Analysis

The assays were conducted in triplicate and across independent experiments. Data from the biofilm assays were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test, with a significance level set at 5%. For the acute toxicity assay, the log-rank test was employed, with a significance threshold of $p < 0.005$.

3. Results

3.1. Antifungal Activity of AM-35 Against Planktonic Cells

The compound AM-35 was tested for its ability to inhibit the fungal growth of *Candida tropicalis* ATCC 750 and *Candida albicans* strain MYA2876. The Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC) values of AM-35, Hydrogel, and Hydrogel+AM-35, as well as the original molecules chalcone and 1% sodium hypochlorite, were determined against oral yeasts. The MIC values of AM-35 were 7.8 µg/mL for *C. albicans* and 3.9 µg/mL for *C. tropicalis*. The MFC values were 7.8 µg/mL for both strains, suggesting that AM-35 is a potent yeast inhibitor. The MFC/MIC ratio indicated that AM-35 has predominantly fungicidal activity against these strains. Additionally, the selectivity index, which was 32 for *C. tropicalis* and 16 for *C. albicans*, demonstrates that the compound exhibits significant fungal selectivity (Table 1).

Table 1. MIC and MFC values of original molecules chalcone, AM-35, Hydrogel containing AM-35, Hydrogel pure and Sodium hypochlorite 1% (standard drugs) against *C. tropicalis* ATCC 750 and *C. albicans* MYA 2876.

Compounds	*C. tropicalis* (ATCC 750 (μg/mL)				*C. albicans* MYA2876 (μg/mL)			
	MIC	MFC	MFC/MIC	SI	MIC	MFC	MFC/MIC	SI
Original Chalcone	>250	>250	-	-	>250	>250	-	-
AM-35	3.9	7.8	2	32	7.8	7.8	1	16
Hydrogel+AM-35	1.95	3.9	2	32	3.9	3.9	1	16
Hydrogel (pure)	125	125	-	-	125	125	-	-
Sodium Hypochlorite (control)	1%	1%	-	-	1%	1%	-	-

3.2. Time-Kill Assay Results

In the time-kill assay, *Candida albicans* and *C. tropicalis* were treated with AM-35, Hydrogel+AM-35, and hydrogel at MFC values. As shown in Figure 1, the fungal cell counts of *C. tropicalis* and *C. albicans* treated with AM-35 or H+AM35 showed a reduction after 6 h of incubation. On the other hand, treatment with hydrogel alone did not show a decrease in the number of yeast cells.

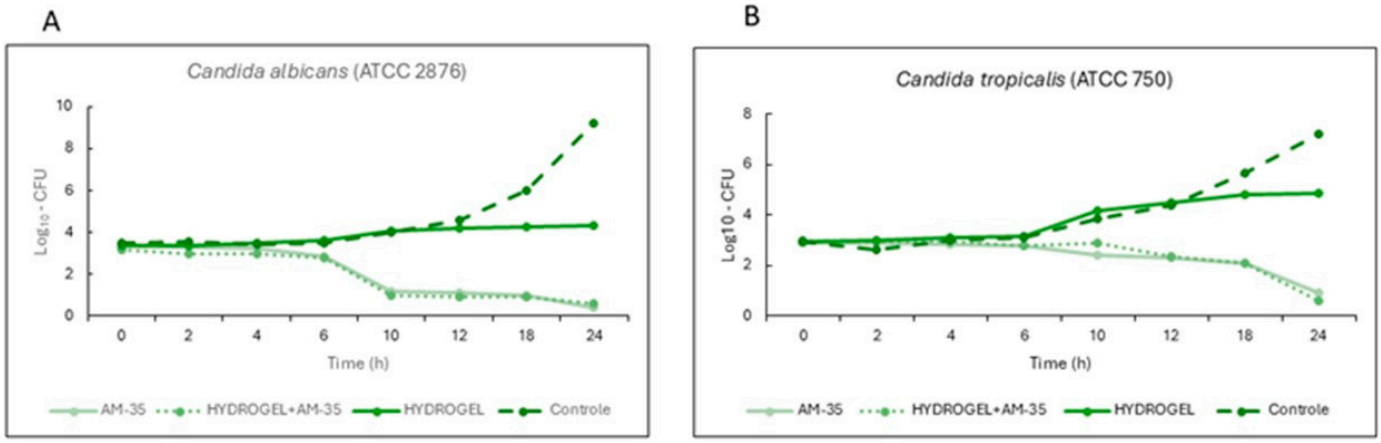

Figure 1. Effect of AM-35 on time-kill curves. (**A**) *Candida albicans* (ATCC 2876) and (**B**) *Candida tropicalis* (ATCC 750) for 24 h exposure. Compounds were tested at a concentration equal to the minimum fungicidal concentration (MFC).

3.3. Hydrogel Containing AM-35 in Single and Mixed Biofilms

Hydrogel containing AM-35 was tested for its ability to inhibit biofilm formation on acrylic resin discs (Figure 2). Treatment with hydrogel containing AM-35 (MIC) and (5xMIC) decreased *C. albicans* and *C. tropicalis* biofilm formation (single and mixed) as compared with the untreated group ($p < 0.05$) by quantification of the colony-forming units (CFU/mL). Furthermore, there was no difference in biofilm formation after treatment with the 1% sodium hypochlorite, as compared with hydrogel containing AM-35 (5xMIC) on biofilm formation.

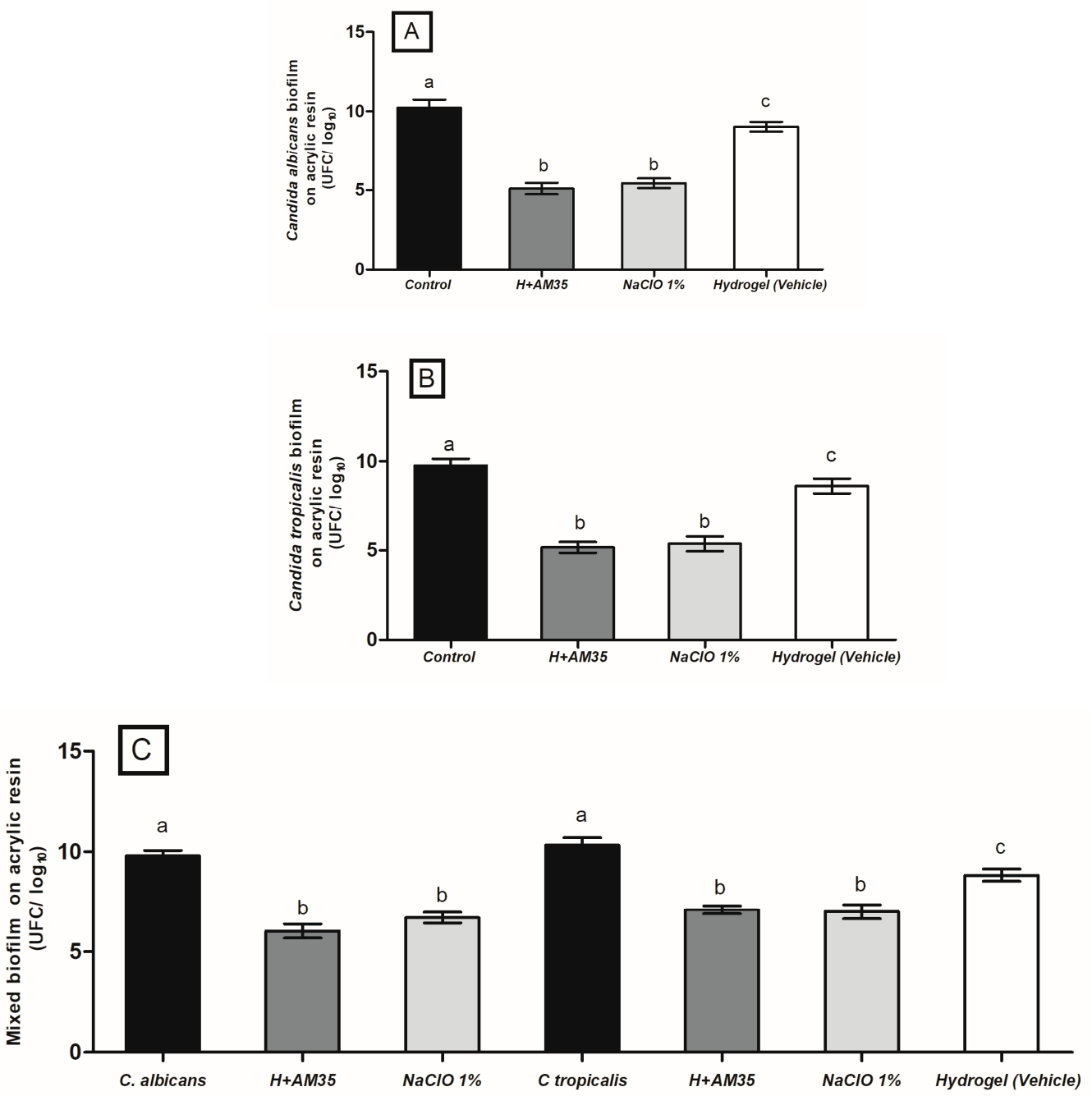

Figure 2. Quantitative analysis (colony-forming units/mL) of the inhibitory effects of hydrogel containing AM-35 (H+AM35) and 1% sodium hypochlorite (NaClO 1%) on *Candida albicans*, Candida tropicalis, and mixed-species biofilm formation on acrylic resin surfaces. (**A**) Treatment with H+AM35 at 5×MIC (39 µg/mL) for *C. albicans*; (**B**) Treatment with H+AM35 at 5×MIC (19.5 µg/mL) for *C. tropicalis*; (**C**) Treatment with H+AM35 at 5×MIC (39 µg/mL) for mixed biofilm. In panel (**C**), the mixed biofilm was quantified separately for *C. albicans* and *C. tropicalis* using CHROMagar™ Candida, which allows differential colony identification. The total biofilm count corresponds to the sum of both species in the control group. All groups were compared statistically, and different letters indicate significant differences (one-way ANOVA followed by Tukey's post-test, $p < 0.05$).

3.4. Results of Acute Systemic Toxicity in Galleria mellonella

Toxicity was assessed using the *G. mellonella* alternative model to verify acute toxicity, as previously described. As seen in Figure 3, the larvae treated with H+AM-35 (5×MIC), which corresponds to the dose of 1.56 mg/kg/larvae, presented low toxicity, maintaining the viability of around 90% of the larvae.

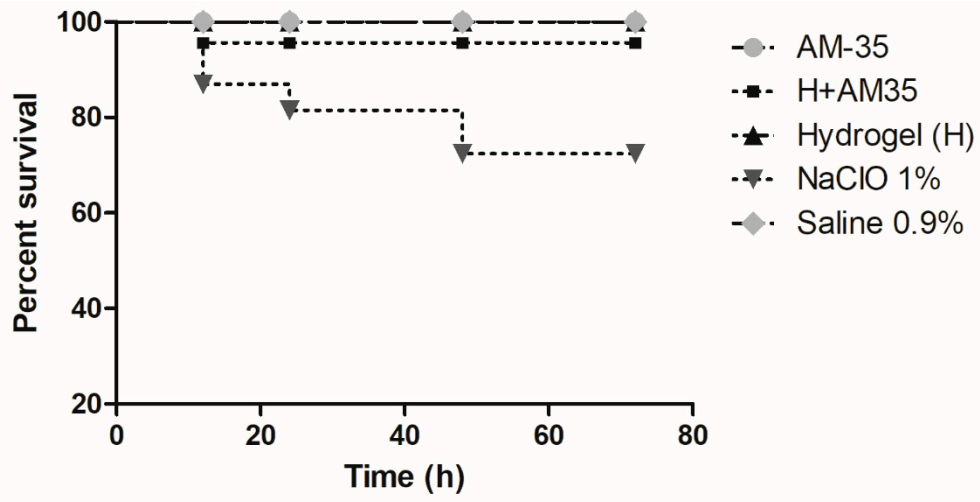

Figure 3. Percentage survival over time of *Galleria mellonella* larvae injected with H+AM-35 at 5×MIC (1.56 mg/Kg/larvae), which corresponds to their effective antibiofilm concentrations previously determined in vitro ($p < 0.05$, log-rank tests).

4. Discussion

Candida is a fungal genus of significant clinical importance, capable of causing a wide range of infections, from superficial to systemic, and represents a considerable challenge in clinical management due to its ability to form biofilms and its increasing resistance to antifungal agents [26]. Topical administration of antimicrobial agents is widely recognized as the most effective approach to maintain active drug concentrations in the oral cavity [27]. The gels can significantly prolong residence time in the oral cavity and hence improve therapeutic effect. A major difficulty for the successful eradication of fungal infections of the oral cavity is the dilution and rapid elimination of topically applied drugs due to the flushing action of saliva [28,29]. The delivery system in which the drug is incorporated is therefore an important consideration and should be formulated to prolong retention of the drug in the oral cavity. The chitosan hydrogel exhibits bioadhesive properties and high viscosity, allowing it to remain in the oral cavity and release the drug over an extended period, thereby enhancing the clinical effect [28].

Thus, this study aimed to use the hydrogel containing aminochalcone to treat infections caused by *Candida* species. The results obtained in this study demonstrate that aminochalcone incorporated into the hydrogel showed promising results in planktonic cells of *Candida albicans* and *C. tropicalis*, as well as in mono-species and mixed-species biofilms of both species.

In antimicrobial activity studies, AM-35 demonstrated significant fungicidal activity, with a Minimum Inhibitory Concentration (MIC) of 3.9 µg/mL for *C. tropicalis* and 7.8 µg/mL for *C. albicans*. Notably, when incorporated into a hydrogel, these MIC values were further reduced, suggesting a synergy with the chitosan present in the hydrogel. Chitosan, a natural polymer, is widely recognized for its potent antifungal activity. Our study is aligned with that of Lo and colleagues (2020) [30], who demonstrated superior efficacy by combining fluconazole with chitosan. Although the hydrogel was initially designed solely as a carrier, its association with AM-35 resulted in a significant improvement in antimicrobial activity.

The adhesion and colonization of microorganisms on surfaces such as mucous membranes and dental prostheses are influenced by factors such as salivary flow, pH, temperature, osmolarity, antimicrobial agents, and host immunity [31]. The positive charge and high surface energy of acrylic resin also facilitate this adhesion by attracting negatively charged salivary molecules that form a film similar to dental enamel. This film creates a

favorable environment for the initial attachment of microorganisms, mediated by adhesins and Van der Waals forces. Regarding mono- and dual-species biofilms, a reduction comparable to that achieved with 1% sodium hypochlorite was observed. Although sodium hypochlorite, in concentrations of 0.5%, 1%, and 5.25%, is effective in removing *Candida* yeast species, its frequent use can cause damage to acrylic resin prostheses, as well as leave an unpleasant residual taste. For these reasons, sodium hypochlorite is not recommended for continuous use.

The time-kill kinetics assay revealed the significant efficacy of the compounds AM-35 and H+AM-35 in combating the growth of *C. albicans* and *C. tropicalis*. After 10 h of exposure, a remarkable fungicidal effect was observed, with approximately 95% of the treated yeast cells being eliminated within 24 h. In comparison, the hydrogel used alone demonstrated a more modest action. Although the chitosan present in the hydrogel has antimicrobial activity, it is possible that the amount used is not sufficient to exert a potent effect on *Candida* strains. This might explain why the hydrogel, when applied alone, showed only a slight effect in combating these yeasts. These results are particularly relevant given the growing clinical interest in fungi and the urgent need to better understand the fungicidal properties and pharmacodynamic characteristics of new antifungal agents. In the biofilm assay, the compounds proved to be even more promising: treatment with H+AM-35 reduced cell viability by 50% (equivalent to a 5-log reduction) for both tested strains. These data highlight the potential of AM-35 and H+AM-35 as effective treatments against fungal infections, especially in contexts where biofilm formation hinders pathogen eradication.

Here, we proposed the use of a new hydrogel containing aminochalcone for the treatment of oral candidiasis. Chalcone is known for its antimicrobial activity [23,25,32,33]. During the study, we were able to demonstrate the action of the hydrogel containing aminochalcone against the biofilm of *C. albicans* and *C. tropicalis*, showing results equivalent to 1% sodium hypochlorite. Regarding in vivo toxicity, the hydrogel showed no toxic effect.

Thus, with the results obtained in this research, we were able to demonstrate the effectiveness of an aminochalcone-based hydrogel as a treatment for oral candidiasis; however, it is important to emphasize the need for continued studies in other models to confirm the efficacy of this antimicrobial hydrogel.

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