

Article

A Silver (I) Complex-Loaded Lipid Nanoemulsion: A New Approach Against *Candida albicans* Biofilms

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Abstract

Introduction: The increasing prevalence of fungal infections, particularly those caused by *Candida albicans*, presents a significant clinical challenge due to the emergence of drug-resistant strains. Silver (I) coordination complexes show promise as antifungal agents; however, their poor water solubility limits clinical application. **Methods:** In this study, we developed and characterized a lipid nanoemulsion (Ag-LN) to enhance the delivery and activity of a silver (I) complex. **Results:** The formulation exhibited nanoscale size, spherical morphology, and stability for up to 60 days. Ag-LN showed potent antifungal effects, preventing biofilm formation and eradicating mature biofilms. Importantly, nanoencapsulation preserved antifungal activity while reducing mutagenic potential and acute toxicity compared with the free compound. **Conclusions:** These findings support Ag-LN as a promising antifungal platform for future preclinical studies.

Keywords: *Candida albicans*; lipid nanoemulsion (Ag-LN); antifungal activity; mutagenicity; biofilms; *Galleria mellonella*



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1. Introduction

Fungal infections are a growing global health problem, causing millions of deaths each year and a troubling increase in antifungal resistance. *Candida albicans* is particularly problematic because it can form biofilms, which provide high tolerance to standard treatments. Although four main classes of antifungals—polyenes, azoles, allylamines, and echinocandins—are available, their effectiveness is limited, highlighting the urgent need for new strategies [1].

Biofilm formation by *C. albicans* is one of the most challenging aspects of fungal infections due to its natural resistance to antifungal drugs and host immune defenses [2]. These biofilms are made up of yeast cells and hyphae embedded in a complex extracellular

matrix (ECM), mainly consisting of polysaccharides, proteins, and nucleic acids, which impedes drug penetration and shields sessile cells from pharmacological and immune system attacks [3].

Furthermore, metabolic heterogeneity and the presence of dormant persister cells within the biofilm further contribute to therapeutic failure and recurrent infections [4–6].

Silver (I) coordination complexes have become promising antifungal agents with multiple mechanisms of action, including disrupting cell membranes, inducing oxidative stress, and interacting with DNA and essential proteins [7,8]. These compounds also inhibit filamentation and biofilm formation, indicating potential to overcome traditional resistance mechanisms. However, their therapeutic use is limited by low aqueous solubility and poor bioavailability [9]. Lipid-based nanocarriers, especially lipid nanoemulsions (LNs), provide an effective way to address these limitations by enhancing solubility, stability, and controlled drug delivery while reducing systemic toxicity [10]. Incorporating silver (I) complexes into such nanostructured systems can improve their ability to penetrate biofilm matrices and enhance antifungal effectiveness against *C. albicans* [11].

In this study, we developed and characterized a lipid nanoemulsion (Ag-LN) loaded with the silver (I) complex $[\text{Ag}(\text{PhTSC} \cdot \text{HCl})_2](\text{NO}_3) \cdot \text{H}_2\text{O}$ and assessed its antifungal activity against *Candida* spp., focusing on biofilm inhibition and eradication. This approach offers an innovative nanoplatform to address current challenges in antifungal therapy.

2. Results

2.1. Silver Coordination Compound $[\text{Ag}(\text{PhTSC} \cdot \text{HCl})_2](\text{NO}_3) \cdot \text{H}_2\text{O}$

The silver (I) coordination compound $[\text{Ag}(\text{PhTSC} \cdot \text{HCl})_2](\text{NO}_3) \cdot \text{H}_2\text{O}$ was synthesized as described in the Materials and Methods section and then used for preparing lipid nanoemulsions. Figure 1 shows the schematic of the synthetic route.

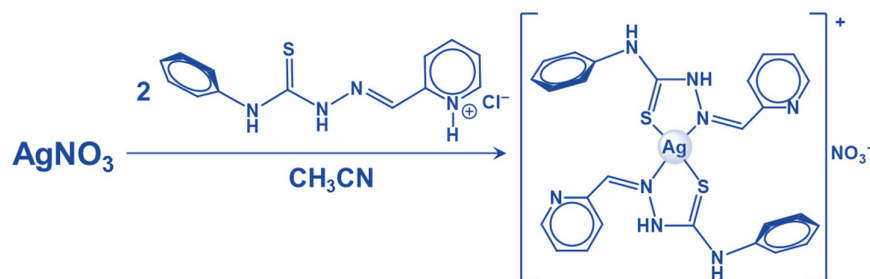


Figure 1. Schematic representation of the synthesis of $[\text{Ag}(\text{PhTSC} \cdot \text{HCl})_2](\text{NO}_3) \cdot \text{H}_2\text{O}$.

2.2. Development of Lipid Nanoemulsion (NL)

Thirty-six different formulations were developed based on a previous study by our research group [4]. Among these, a lipid nanoemulsion (LN) was selected for characterization and antibiofilm activity testing. This formulation consisted of a 10% oil phase (grape seed oil and cholesterol, in a 1:1 ratio), an 80% PBS (aqueous phase, pH 7.4), and a 10% surfactant mixture of Brij 35/SPA (2:1). The LN was sonicated at 500 W for 20 min in pulsed mode (with 30 s intervals every minute) using an ice bath. The resulting nanoemulsions were centrifuged at $11,180 \times g$ (approximately ≈ 9.530 rpm, rotor radius = 11.0 cm) for 15 min.

The different regions identified were visually classified as viscous or liquid, transparent, translucent, opaque, or phase-separated systems. Based on this classification, a ternary phase diagram of the CHO/GSO–SPC/Brij 35–PBS system was created (Figure 2). This diagram helped identify the isotropic liquid transparent region (LTS), from which the formulation was chosen for incorporating the Ag(I) coordination complex.

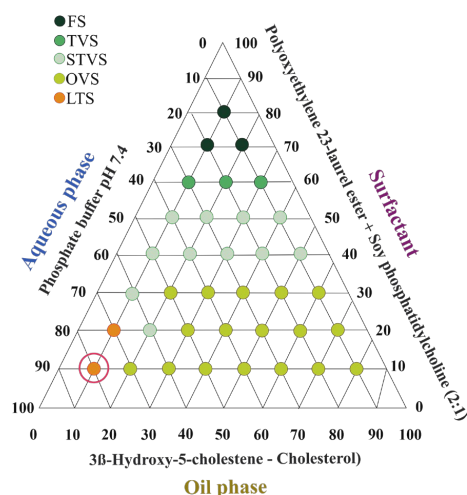


Figure 2. Ternary phase diagram of the system composed of PBS (pH 7.4), cholesterol + grape seed oil (1:1, *w/w*), and SPC/Brij[®] 35 (1:2, *w/w*). The different regions were visually classified into five categories: FS (fluid system), TVS (translucent viscous system), STVS (semi-translucent viscous system), OVS (opaque viscous system), and LTS (liquid transparent system). The circled point indicates the selected composition for the preparation of lipid nanoemulsions loaded with the Ag(I) coordination complex.

2.3. Physicochemical Characterization of NLs

2.3.1. Size and Polydispersity Index

The hydrodynamic diameter and polydispersity index (PDI) of B-NL and Ag-LN were measured using dynamic light scattering (DLS). All nanoemulsions (LN) created in this study had sizes below 200 nm, with hydrodynamic diameters of 61.00 ± 1.674 nm for B-NL and 112.57 ± 1.082 nm for Ag-LN, showing their potential as drug delivery nanosystems. PDI values of 0.261 ± 0.010 (B-NL) and 0.248 ± 0.007 (Ag-LN) indicate relatively narrow size distributions, matching literature standards for monodispersity. The PDI is a dimensionless measure of particle size distribution, ranging from 0 to 1; values below 0.2 denote high uniformity (monodisperse systems), while values closer to 1 signify broader distributions (polydispersity) [12]. DLS measures particle size by assessing Brownian motion in solution and estimating dimensions based on light scattering, including the solvation layer surrounding nanoparticles. Overall, the results confirm that the nanoemulsions have suitable sizes for controlled drug release. Meanwhile, the PDI values indicate a high degree of size uniformity, meeting key requirements for stable and effective nanosystems.

2.3.2. Zeta Potential (ZP)

Zeta potential (ZP) is a crucial measure of colloidal stability, indicating the level of electrostatic repulsion or attraction between particles. High absolute ZP values generally mean better stability, as strong repulsive forces among similarly charged particles prevent aggregation. Colloidal nanosystems with ZP values around ± 30 mV are usually considered stable [13,14]. In this study, the measured ZP values were -3.39 ± 0.605 mV for B-NL and -3.02 ± 0.503 mV for Ag-LN. Although these values are below the conventional threshold, the formulations are likely stable because of the steric effects provided by the surfactants, which help maintain proper dispersion in the medium. Table 1 summarizes the results of hydrodynamic diameter, polydispersity index (PDI), and zeta potential analyses.

2.3.3. Stability Assessment of B-LN and NL Ag-LN

To evaluate the stability of the nanosystems, the colloidal properties of B-LN and Ag-LN were measured at different time points: 24 h after synthesis (time 1), and after 7,

15, 30, 45, and 60 days. Figure 3A shows the particle size data for B-LN and Ag-LN, with no statistically significant differences observed over 60 days ($p > 0.05$). The hydrodynamic diameter (PDI) analysis (Figure 3B) also indicated no significant differences between days 7 and 45 compared to day 1 ($p > 0.05$), confirming stability during this period. From days 15 to 60, Ag-LN remained stable in size. However, a significant change ($p < 0.05$) in ZP was observed between days 1 and 7, with no further differences at later time points (Figure 3C). The LN formulation remained stable regarding ZP. Overall, the findings suggest that variations could occur after 60 days, which is an essential consideration for applications requiring extended stability.

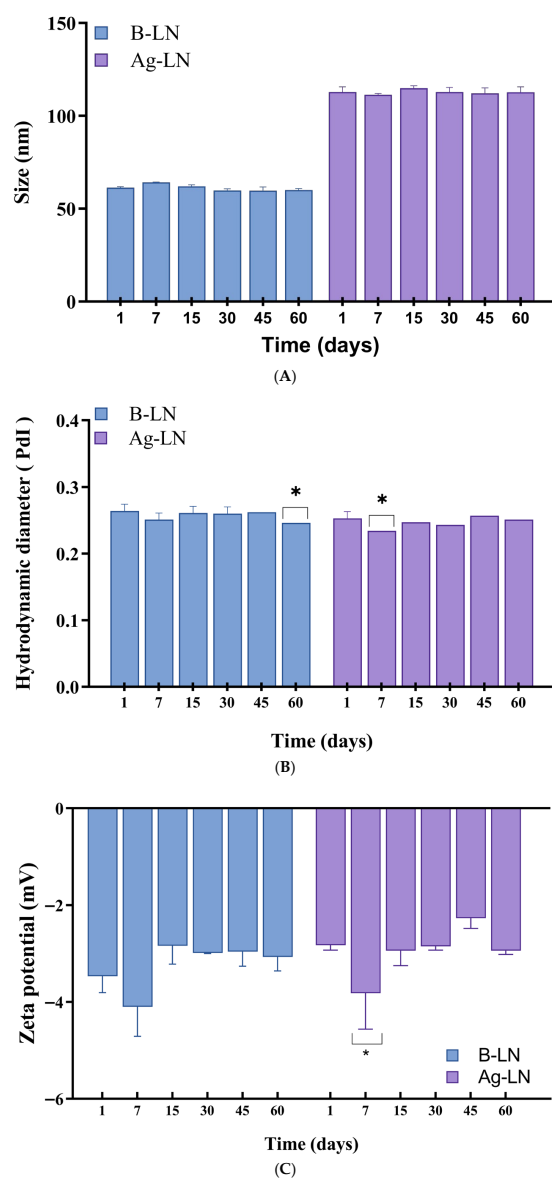


Figure 3. (A) Mean Hydrodynamic Diameter (Z-Ave) of B-LN and Ag-LN. Particle size (nm) of B-LN and Ag-LN at different time points. $[\text{Ag}(\text{PhTSC} \cdot \text{HCl})_2](\text{NO}_3) \cdot \text{H}_2\text{O}$. Data are expressed as mean $\pm$ SD. Statistical analysis was performed by two-way ANOVA followed by Dunnett's multiple comparisons test. (B) Polydispersity Index (PDI) of B-LN and Ag-LN. Polydispersity Index (PDI) of B-LN and Ag-LN over time. $[\text{Ag}(\text{PhTSC} \cdot \text{HCl})_2](\text{NO}_3) \cdot \text{H}_2\text{O}$. Data are expressed as mean $\pm$ SD. Statistical analysis was performed by two-way ANOVA followed by Dunnett's multiple comparisons test; (*) $p < 0.05$. (C) Zeta Potential (mV) of B-LN and Ag-LN. Zeta potential (mV). $[\text{Ag}(\text{PhTSC} \cdot \text{HCl})_2](\text{NO}_3) \cdot \text{H}_2\text{O}$. Data are expressed as mean $\pm$ SD. Statistical analysis was performed by two-way ANOVA followed by Dunnett's multiple comparisons test; (*) $p < 0.05$.

Table 1. Dynamic light scattering (DLS) analysis of blank nanoemulsion (B-NL) and Ag(I)-loaded nanoemulsion (Ag-LN). Data are expressed as mean $\pm$ standard deviation.

Samples	Size (nm)	PDI	ZP (mV)
B-LN	61.00 $\pm$ 1.6	0.26 $\pm$ 0.01	−3.39 $\pm$ 0.605
Ag-LN	112.57 $\pm$ 1.0	0.24 $\pm$ 0.00	−3.02 $\pm$ 0.5

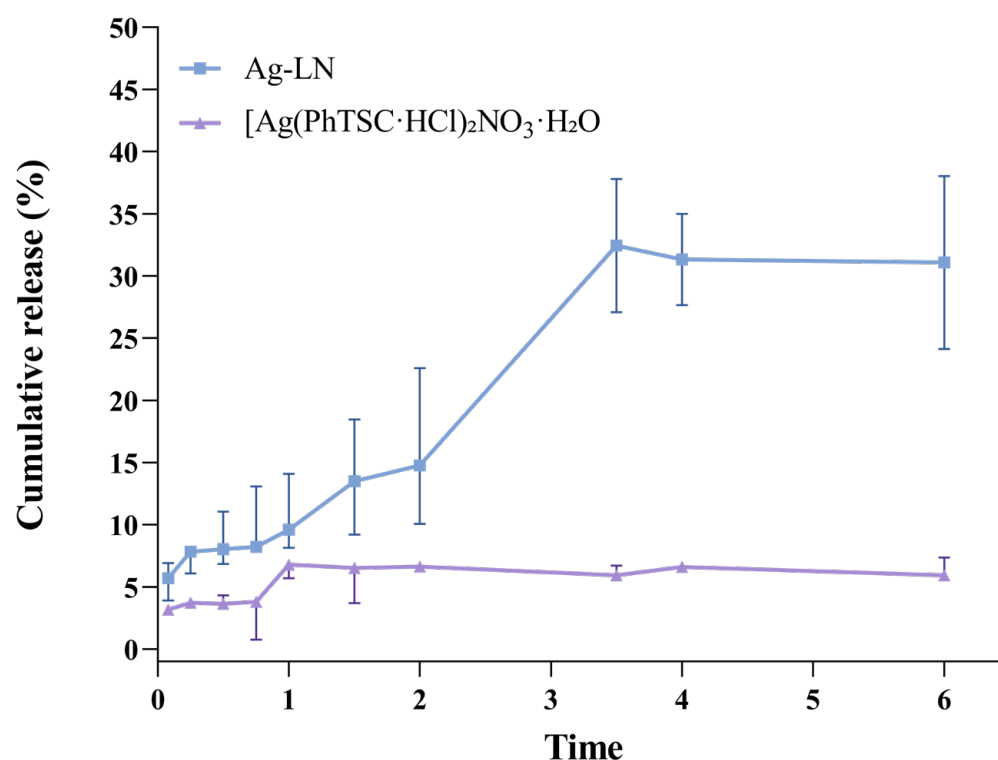
Abbreviations: nm, nanometer; mV, millivolt; PDI, polydispersity index; ZP, zeta potential; B-LN, (Blank Lipid Nanoemulsion); Ag-LN (Silver-loaded Lipid Nanoemulsion).

2.4. In Vitro Drug Release

The drug release profile from nanostructured systems is a key factor for assessing the effectiveness and performance of the developed formulation. These systems hold the drug during systemic circulation, ensuring that a substantial portion of the administered dose reaches its therapeutic site of action [15,16].

Furthermore, controlled-release profiles decrease fluctuations in plasma concentrations, preventing both toxic peaks and subtherapeutic levels, thereby improving clinical efficacy and safety. Prolonged-release systems also lower the frequency of dosing, enhance patient convenience, and support treatment adherence [15,17].

In this context, researchers evaluate in vitro release kinetics (Figure 4) as a critical step in pharmaceutical development, because it provides insights to predict in vivo behavior and to guide the selection of the most suitable formulation for the intended therapeutic purpose [16].

**Figure 4.** In vitro cumulative release profile (%) of the silver coordination complex from lipid nanoemulsions (Ag-LN) compared with the blank nanoemulsion (B-NL) at pH 6.8, over 6 h. Data are expressed as mean $\pm$ SD (n = 3).

At pH 6.8, which mimics the microenvironment where *Candida albicans* proliferates and forms biofilms, the free silver compound exhibited limited release, stabilizing at approximately 6% up to 6 h. In contrast, the silver-loaded lipid nanoemulsion (Ag-LN) displayed a biphasic and sustained release pattern. The system exhibited an initial diffusion-driven

phase (5–120 min; excellent fit to the Higuchi model, $R^2 \approx 0.98$; Korsmeyer–Peppas $n \approx 0.32$, consistent with Fickian diffusion), followed by an accelerated phase after approximately 3 h, reaching around 40% release at 6 h. This behavior demonstrates that the nanostructured carrier maintained silver solubility for extended periods, resulting in approximately sixfold higher release compared with the free compound.

2.5. Transmission Electron Microscopy (TEM)

Transmission electron microscopy (TEM) was conducted as part of the physicochemical characterization of the lipid nanoemulsions. TEM micrographs (Figure 5A–D) show spherical droplets with a uniform distribution for both B-NL and Ag-LN, with no signs of aggregation. The observed sizes appear to be below 200 nm, consistent with the DLS data (Table 1). Ag-LN exhibits higher electron contrast compared to B-NL, which aligns with the presence of silver-containing species. Incorporation of $[\text{Ag}(\text{PhTSC} \cdot \text{HCl})_2](\text{NO}_3) \cdot \text{H}_2\text{O} \cdot \text{H}_2\text{O}$ did not alter droplet morphology, aligning with reports that drug loading in lipid nanocarriers preserves spherical shape and internal organization [18–21].

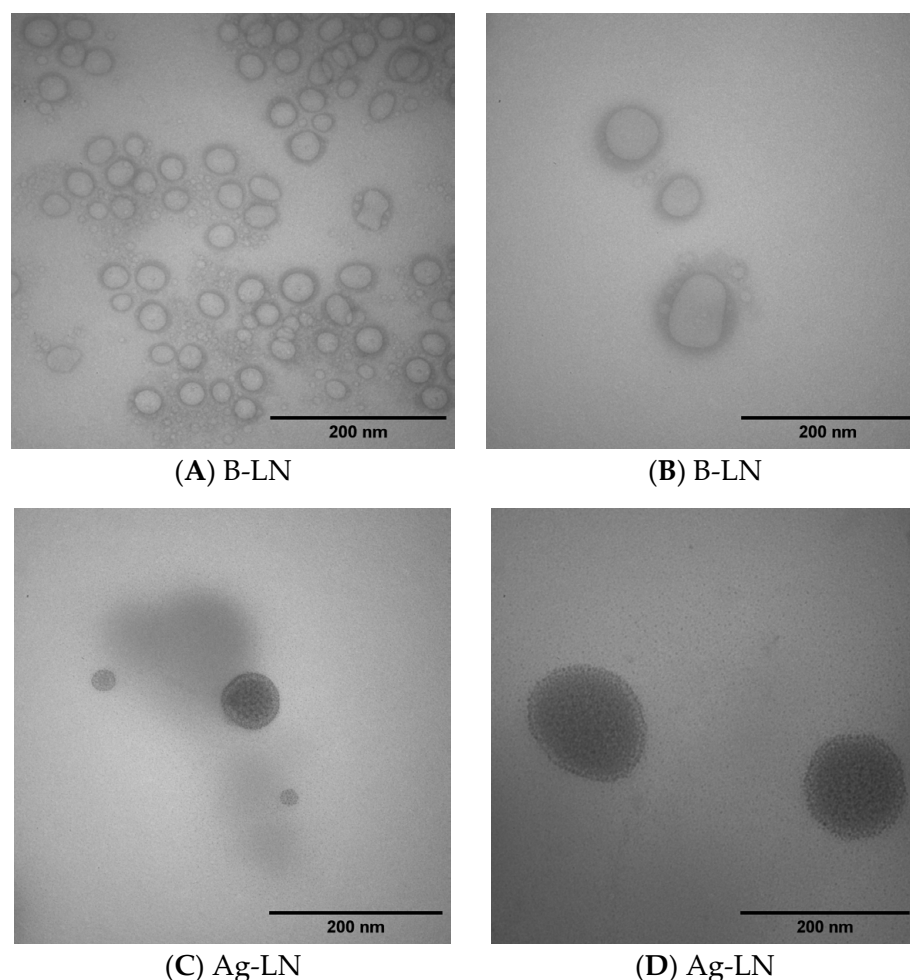


Figure 5. TEM images of the blank nanoemulsion (B-NL): (A) 100 k $\times$ and (B) 200 k $\times$; and the silver-loaded nanoemulsion (Ag-LN): (C) 100 k $\times$ and (D) 200 k $\times$. Droplets are mostly spherical, with no visible aggregation, and maintain a consistent sub-200 nm size. Ag-LN exhibits higher electron contrast than B-NL.

2.6. Rheological Test

2.6.1. Flow Rheology

Rheological analysis was performed for B-NL and Ag-LN formulations. Both systems exhibited non-Newtonian behavior, as indicated by the non-linear relationship between shear stress and shear rate (Figure 6A,B). This behavior is characteristic of systems in which viscosity varies with applied force, which is common in complex formulations such as B-NL.

2.6.2. Oscillatory Rheology

Oscillatory rheological analysis indicated that both formulations exhibit thixotropic behavior. The storage modulus (G') exceeded the loss modulus (G'') in both samples, indicating predominance of elastic over viscous behavior. For the B-NL formulation, G' was 449.84 Pa and G'' was 34.69 Pa; for Ag-LN, G' was 978 Pa and G'' was 121.5 Pa (Figure 6C,D).

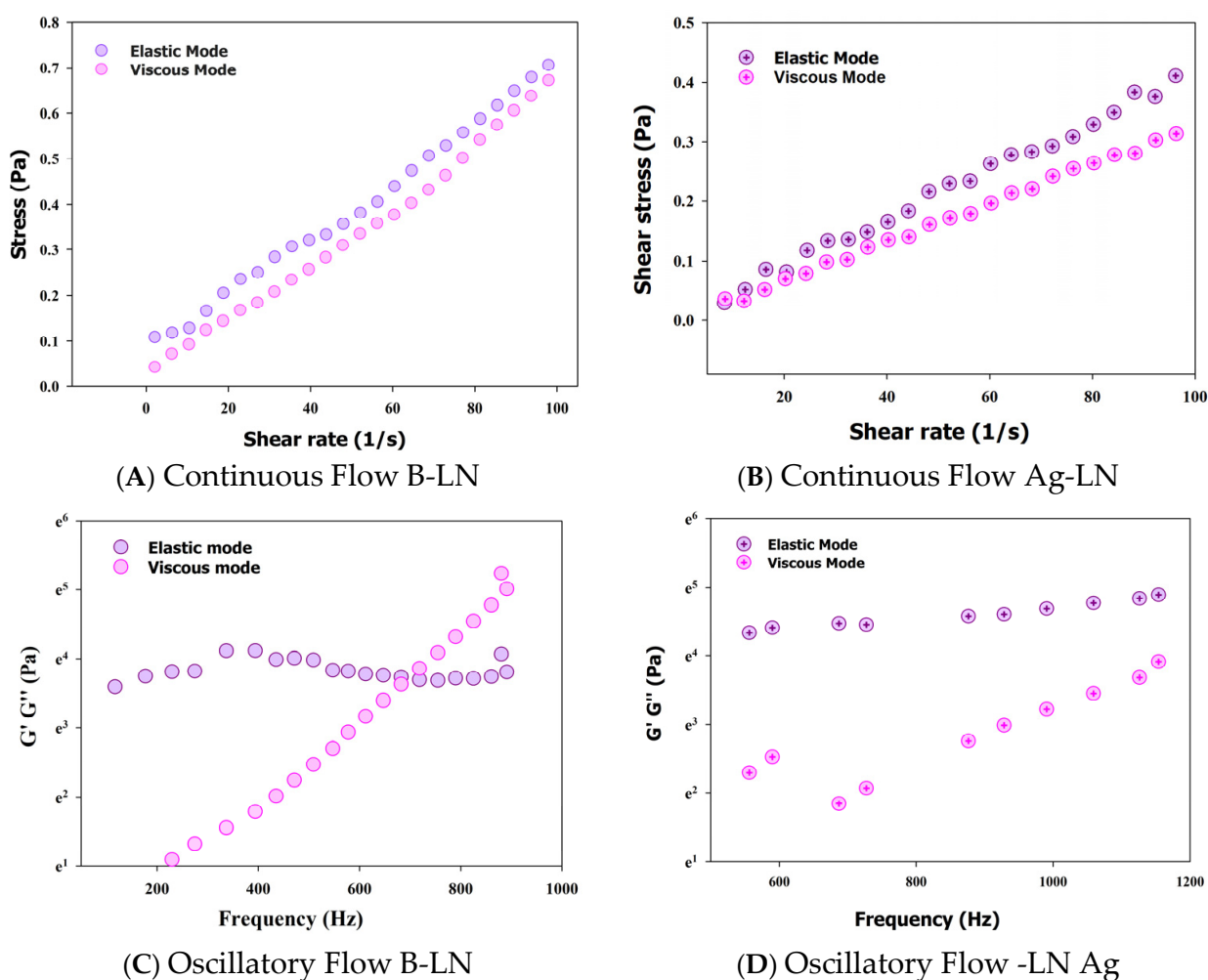


Figure 6. Rheograms of Continuous flow and oscillatory Flow for B-LN and Ag-LN. Continuous and oscillatory rheology graphs; B-NL: Blank lipidic nanoemulsion; Ag-LN: Nanoemulsion + $[\text{Ag}(\text{PhTSC} \cdot \text{HCl})_2](\text{NO}_3) \cdot \text{H}_2\text{O}$.

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

Table 2 presents the Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC) values of the tested samples against standard Candida strains. The free silver coordination compound $[\text{Ag}(\text{PhTSC} \cdot \text{HCl})_2](\text{NO}_3) \cdot \text{H}_2\text{O}$ exhibited the lowest

MIC and MFC values against *C. albicans* ATCC 10,231 (3.9 µg/mL and 7.8 µg/mL, respectively). The nanostructured formulation Ag-LN also showed significant activity, with MIC and MFC values of 15.6 µg/mL, indicating that incorporation of the compound into the nanoemulsion did not compromise its efficacy.

In addition to the complete formulations, the thiosemicarbazone ligand (TSC) and silver nitrate (AgNO₃), which are components of the coordination complex, were tested individually. Silver nitrate exhibited MIC and MFC values of 0.24 µg/mL against *C. albicans* ATCC 10231, while isolated TSC showed MIC and MFC values of 1.9 and 3.9 µg/mL, respectively. The positive controls, amphotericin B (AMB) and fluconazole (FLU), displayed variable activity across strains, with FLU being ineffective against some of them. The blank nanoemulsion (B-NL) and the DMSO solvent showed no antifungal activity at the tested concentrations.

Table 2. Minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of the evaluated compounds.

Samples:	<i>C. albicans</i> (ATCC 10231)	<i>C. glabrata</i> (ATCC 2001)	<i>P. kudriavzevii</i> (ATCC 6528)	<i>C. parapsilosis</i> (ATCC 2219)	<i>C. tropicalis</i> (ATCC 750)
µg/mL	MIC/MFC	MIC/MFC	MIC/MFC	MIC/MFC	MIC/MFC
B-LN	-	-	-	-	-
AMB	0.12	0.25	0.50	0.50	0.50
FLU	R	R	R	16	128
TSC	1.9/3.9	7.8/FS	0.48/0.48	7.8/31.25	31.25/FS
AgNO ₃	0.24/0.24	0.97/1.9	0.48/0.48	0.97/1.9	0.97/1.9
DMSO	-	-	-	-	-
Ag-LN	15.6/15.6	15.6/62.5	31.25/31.25	31.25/31.25	31.25/62.5
[Ag(PhTSC·HCl) ₂](NO ₃)·H ₂ O	3.9/7.8	3.9/7.8	7.8/7.8	7.8/15.6	15.6/31.25

B-NL (blank lipid nanoemulsion); AMB (amphotericin B); FLU (fluconazole); TSC (thiosemicarbazone ligand); AgNO₃ (silver nitrate); DMSO (solvent control); Ag-LN (lipid nanoemulsion containing the silver coordination compound); [Ag(PhTSC·HCl)₂](NO₃)·H₂O (free silver coordination compound); FS (fungistatic activity). Note: B-NL, AMB, FLU, and DMSO showed no detectable antifungal activity (MFC not determined).

In Vitro Inhibition of Biofilm Formation

The results presented in Figure 7 demonstrate that both the free silver coordination compound [Ag(PhTSC·HCl)₂](NO₃)·H₂O and the nanoemulsion loaded with the compound (Ag-LN) were effective in inhibiting *C. albicans* biofilms. During the biofilm formation phase (24 h), both treatments exhibited near-complete inhibition at concentrations of 2000 and 1000 µg/mL. At lower concentrations, such as 250 µg/mL, the Ag-LN formulation maintained inhibition levels above 70%, indicating its potential as a preventive strategy in the initial stages of biofilm development. This feature may be particularly advantageous in clinical or industrial contexts, such as on medical devices or surfaces prone to fungal contamination.

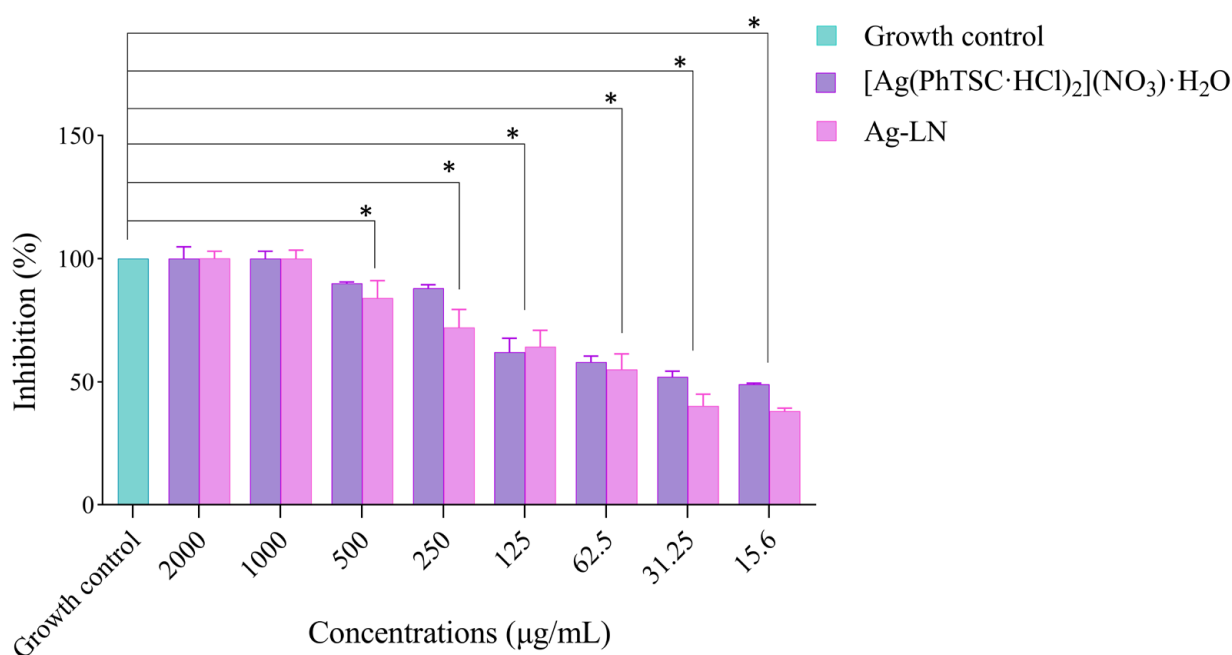


Figure 7. Growth inhibition of $[\text{Ag}(\text{PhTSC} \cdot \text{HCl})_2](\text{NO}_3) \cdot \text{H}_2\text{O}$ compared with silver-loaded lipid nanoemulsion (Ag-LN) at different concentrations (2000–15.6 µg/mL). Growth control corresponds to untreated cells. Data are presented as mean $\pm$ SD ($n = 3$). Statistical analysis was performed by two-way ANOVA followed by Tukey's test; (*) $p < 0.05$.

The results presented in Figure 8 demonstrate that, after 72 h, the free compound $[\text{Ag}(\text{PhTSC} \cdot \text{HCl})_2](\text{NO}_3) \cdot \text{H}_2\text{O}$ exhibited vigorous antifungal activity, achieving 100% inhibition at 2000 µg/mL and approximately 80% at 250 µg/mL. Although the Ag-LN was also effective at higher concentrations, its antifungal efficacy declined more markedly at lower concentrations. At 1000 µg/mL, inhibition decreased to approximately 78%, while activity remained slightly above 70% at 250 µg/mL.

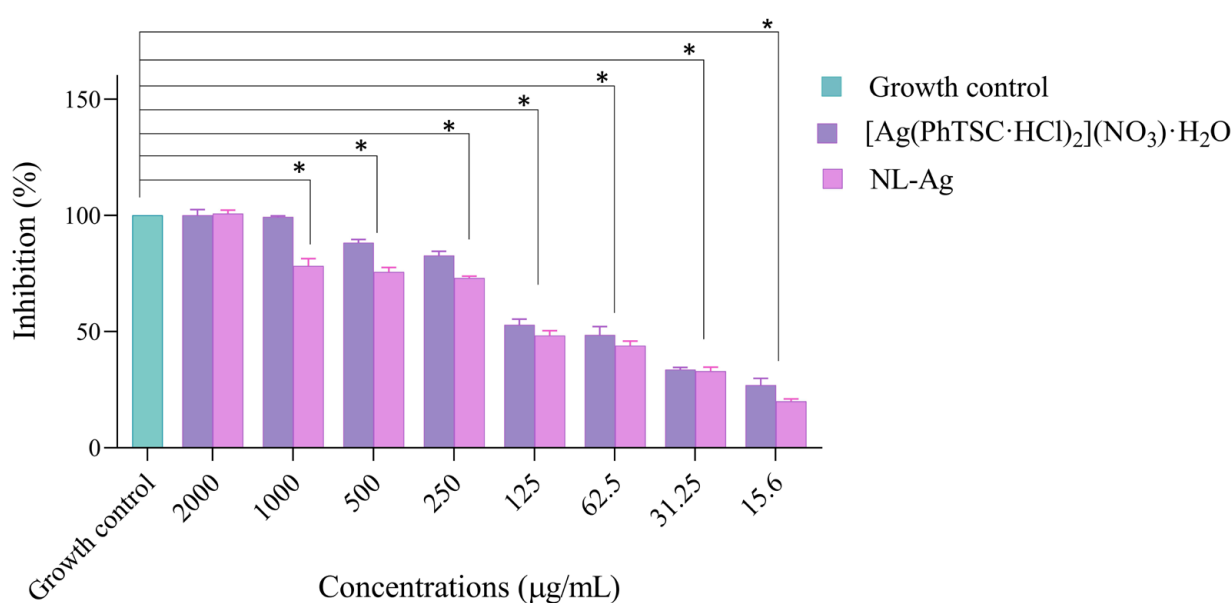


Figure 8. Inhibition of metabolic activity in mature biofilms (72 h) after treatment with the free silver coordination compound. Growth inhibition of $[\text{Ag}(\text{PhTSC} \cdot \text{HCl})_2](\text{NO}_3) \cdot \text{H}_2\text{O}$ compared with silver-loaded lipid nanoemulsion (Ag-LN) at different concentrations (2000–15.6 µg/mL). Growth control corresponds to untreated cells. Data are presented as mean $\pm$ SD ($n = 3$). Statistical analysis was performed by two-way ANOVA followed by Tukey's test; (*) $p < 0.05$.

These findings suggest that, although both formulations exhibit promising activity, the free compound may offer superior efficacy against mature biofilms. This advantage could be attributed to its greater ability to interact directly with the dense extracellular matrix typical of advanced biofilm stages.

2.8. Determination of Mutagenic Potential

To evaluate the mutagenic potential of the silver coordination compound $[\text{Ag}(\text{PhTSC} \cdot \text{HCl})_2](\text{NO}_3) \cdot \text{H}_2\text{O}$ and its nanoemulsion formulation (Ag-LN), the Ames test was conducted (Table 3). Our group previously reported the mutagenicity of the free compound [22]. Table 3 shows the results for Ag-LN, including the average number of revertants per plate (M), standard deviation, and mutagenicity index (MI), after treating *S. Typhimurium* strains TA98, TA100, TA97a, and TA102 with and without metabolic activation (+S9 and −S9, respectively). A sample was considered mutagenic if a dose-dependent response was observed and/or if $\text{MI} \geq 2$ at any tested concentration.

Consistent with previous findings, $[\text{Ag}(\text{PhTSC} \cdot \text{HCl})_2](\text{NO}_3) \cdot \text{H}_2\text{O}$ showed a mutagenic profile, with $\text{MI} \geq 2$ in the TA97a strain under metabolic activation (+S9); additional mutagenic potential was detected in the TA98 strain under the same conditions. Conversely, incorporating the compound into the lipid nanoemulsion eliminated this effect. Under all tested conditions, Ag-LN did not demonstrate mutagenic potential, regardless of the presence or absence of metabolic activation.

Table 3. Mutagenic showed number of reversers/plates, standard and mutagenicity index (MI) of compound and Ag-LN against *S. typhimurium* with (+S9) and without (−S9) metabolic strains TA 98, TA 100, TA 102 and TA97a. $\text{M} \pm \text{SD}$ = mean and standard deviation.

Number of Reversers/Plate (MI) and Mean and Standard Deviation ($\text{M} \pm \text{DP}$)								
Strains:	TA98 (−S9)	TA98 (+S9)	TA100 (−S9)	TA100 (+S9)	TA102 (−S9)	TA102 (+S9)	TA97a (−S9)	TA97a (+S9)
Treatment: Ag-LN ($\mu\text{g}/\text{plate}$)								
0.62	30 ± 3 (0.92)	22 ± 0 (0.96)	110 ± 9 (1.15)	120 ± 5 (1.01)	363 ± 18 (1.03)	326 ± 32 (1.11)	138 ± 11 (1.03)	209 ± 15 (1.37)
1.25	32 ± 4 (0.97)	22 ± 1 (0.96)	112 ± 6 (1.17)	128 ± 16 (1.07)	301 ± 15 (0.86)	300 ± 29 (1.03)	168 ± 13 (1.25)	191 ± 18 (1.25)
2.50	33 ± 4 (1.00)	24 ± 1 (1.04)	112 ± 14 (1.17)	131 ± 11 (1.10)	273 ± 15 (0.78)	288 ± 19 (0.98)	152 ± 11 (1.13)	194 ± 5 (1.27)
3.75	31 ± 4 (0.94)	23 ± 6 (0.98)	114 ± 16 (1.19)	130 ± 6 (1.09)	262 ± 22 (0.75)	338 ± 20 (1.15)	127 ± 17 (0.95)	137 ± 2 (0.90)
5.0	31 ± 1 (0.95)	22 ± 1 (0.96)	115 ± 13 (1.20)	133 ± 8 (1.11)	314 ± 11 (0.90)	268 ± 14 (0.92)	130 ± 13 (0.97)	111 ± 4 (0.73)
Blank	34 ± 3 (1.05)	24 ± 1 (1.02)	139 ± 10 (1.46)	148 ± 10 (1.24)	291 ± 13 (0.83)	308 ± 15 (1.05)	136 ± 16 (1.01)	142 ± 10 (0.93)
C+	857 ± 46 a	925 ± 20 d	1250 ± 157 b	1262 ± 82 d	2288 ± 185 c	1117 ± 78 e	1526 ± 88 a	1015 ± 53 d
C esp.	33 ± 5	23 ± 3	96 ± 5	119 ± 14	351 ± 15	293 ± 20	134 ± 8	153 ± 12

MI = Mutagenicity Index (values in parentheses); Negative control (C−): DMSO, 100 $\mu\text{L}/\text{plate}$; Positive controls (C+): 4-nitro-o-phenylenediamine (NPD, 10 $\mu\text{L}/\text{plate}$, TA98), sodium azide (1.25 $\mu\text{L}/\text{plate}$, TA100), mitomycin C (3 $\mu\text{L}/\text{plate}$, TA102; all without S9), and 2-anthramine (1.25 $\mu\text{L}/\text{plate}$, all strains; with S9); Ag-LN = nanoemulsion containing $[\text{Ag}(\text{PhTSC} \cdot \text{HCl})_2](\text{NO}_3) \cdot \text{H}_2\text{O}$ (silver coordination compound). Values are expressed as mean $\pm$ SD. Different letters (a–e) indicate statistically significant differences among groups ($p < 0.05$).

2.9. Determination of Acute Toxicity Potential in an Alternative Model: Galleria Mellonella

In this study, we assessed the acute toxicity of the silver coordination compound $[\text{Ag}(\text{PhTSC} \cdot \text{HCl})_2](\text{NO}_3) \cdot \text{H}_2\text{O}$, both in its free form. We encapsulated it in a nanoemulsion

(Ag-LN), using *Galleria mellonella* larvae. We based the tested concentrations on the weight of the active compound. Additionally, we included a control group that received the blank nanoemulsion (without the silver compound) to evaluate the potential effects of the formulation itself. Figure 9A,B presents the results, comparing larval survival across different treatments and concentrations.

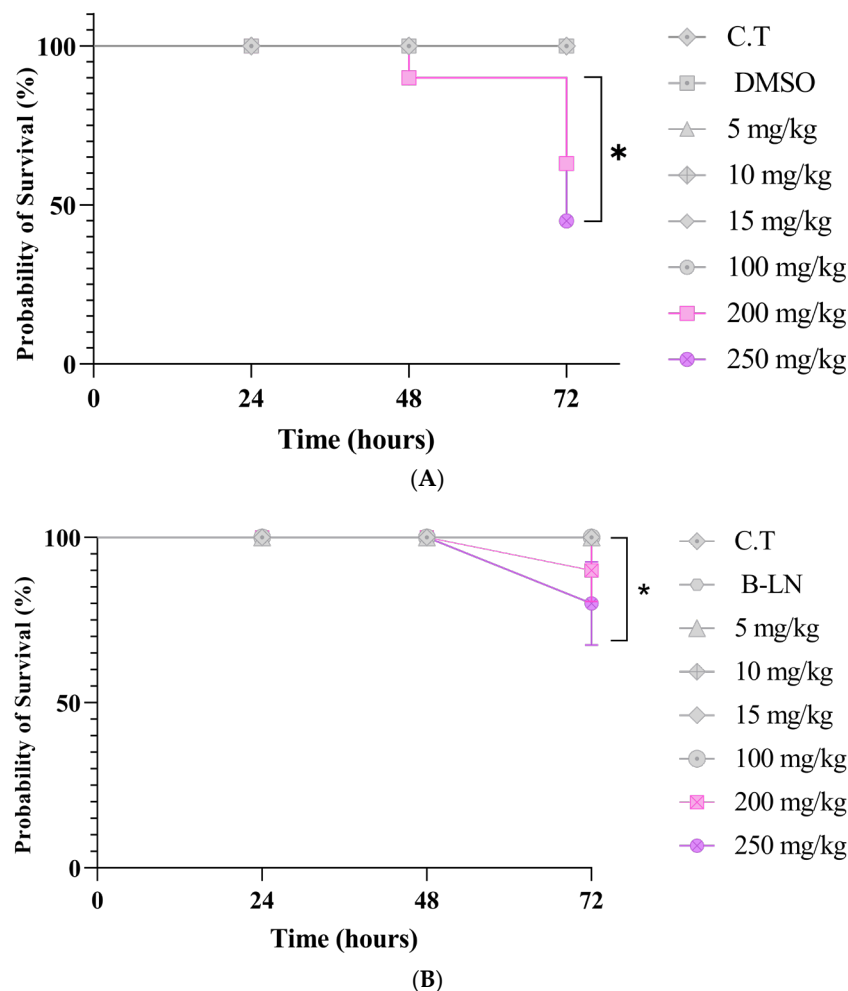


Figure 9. (A) Determination of the acute toxic potential of $[Ag(PhTSC \cdot HCl)_2](NO_3) \cdot H_2O$ using the alternative *Galleria mellonella* model. Values expressed in (%); Trauma Control (CT); Blank Lipid Nanoemulsion (B-NL); silver coordination compound $[Ag(PhTSC \cdot HCl)_2](NO_3) \cdot H_2O$. Statistical analysis was performed using the Log-rank (Mantel–Cox) test, Log-rank test for trend, and Gehan–Breslow–Wilcoxon test. Significant differences between survival curves were observed (* $p < 0.0001$). (B) Determination of the acute toxic potential of Ag-LN using the alternative *Galleria mellonella* model. Values expressed in (%); Trauma Control (CT); Blank Lipid Nanoemulsion (B-NL); Ag-NL: Statistical analysis was performed using the Log-rank (Mantel–Cox) test, Log-rank test for trend, and Gehan–Breslow–Wilcoxon test. Significant differences were observed between the survival curves (* $p < 0.0001$).

3. Discussion

The physicochemical characterization provided essential parameters for assessing the stability and performance of the developed nanoemulsions. The droplet size is a critical parameter in the formation and stability of nanoemulsions [12]. According to Öztürk [23], it has a significant impact on biological outcomes, such as cellular uptake, toxicity, and drug dissolution. In this study, the mean droplet size of the B-NL formulation was 61.00 ± 1.674 nm, while that of Ag-NL was 112.57 ± 1.082 nm. Both values are within the

10–200 nm range, considered optimal for nanoemulsions, confirming the physicochemical suitability and therapeutic potential of the developed nanostructures.

PDI values near 0.2 suggest a monomodal and uniform distribution, which is preferred in nanoparticulate systems because it is linked to greater stability and reproducibility [24].

As described by Wang [25], the zeta potential (ZP) reflects the surface charge of particles and offers insight into the electrostatic forces of repulsion or attraction between them. It is a crucial parameter for evaluating the stability of colloidal systems, affecting aggregation, flocculation, and dispersion.

The negative charge observed in the analyzed systems can be attributed to the presence of phospholipids, such as soy phosphatidylcholine, used in the formulation. This phospholipid imparts a negative potential to the colloidal interface because of the phosphate groups in its lipid structure. According to Silva [26], the dissociation of these groups in water creates a negative surface charge in lipid systems containing phosphatidylcholine, helping to maintain the colloidal stability of the formulations.

To evaluate system stability, the coordination metal compound and Ag-NL were analyzed at different time points: 24 h after formulation (time 1) and at 7, 15, 30, 45, and 60 days. A study by Dadwal et al. developed a nanoemulsion to transport squaric acids, resulting in a nanoemulgel for enhanced delivery of clobetasol propionate to sebaceous glands. After six months, their system showed a droplet size of 240.5 ± 9.2 nm, a PDI of 0.282 ± 0.03 , and a ZP of -51.21 mV, suggesting particle stability [27].

These findings support our study, as our formulations align with stability parameters reported in the literature. Furthermore, our assessment indicates that the systems remain stable for at least 60 days; however, longer-term studies are recommended.

After confirming the physicochemical stability and surface characteristics of the nanoemulsions, the in vitro release behavior of the silver (I) complex was investigated under biofilm-like conditions (pH 6.8), as presented in Figure 4.

Quantitative solubility values (Sapp) for the silver (I) complex in water or PBS were not determined. In this study, the observed restricted release is consistent with poor intrinsic aqueous solubility. In contrast, the presence of Brij® 35 in the nanoemulsion system is expected to enhance apparent solubility through micelle-mediated solubilization, as extensively documented for poorly water-soluble drugs [28–31]. This mechanism helps explain the significantly higher release observed for Ag-LN compared with the free compound.

In line with this reasoning, the silver-loaded lipid nanoemulsion (Ag-LN) showed a biphasic and sustained release pattern. The system exhibited an initial diffusion-driven phase (5–120 min; excellent fit to the Higuchi model, $R^2 \approx 0.98$; Korsmeyer–Peppas $n \approx 0.32$, consistent with Fickian diffusion), followed by an accelerated phase after about 3 h, reaching around 40% release at 6 h. This biphasic profile suggests that the nanostructured carrier keeps silver soluble over extended periods, enabling gradual delivery and resulting in roughly six times higher release compared to the free compound. Such a sustained profile aligns with the post-loading mechanism used for Ag-LN preparation of $[\text{Ag}(\text{PhTSC} \cdot \text{HCl})_2](\text{NO}_3) \cdot \text{H}_2\text{O}$, which was not determined experimentally; its incorporation into pre-formed nanoemulsions depends on a surfactant-rich interfacial layer that promotes solubilization and entrapment of the silver (I) complex during probe sonication [32–34]. Similar methods have been widely used with poorly water-soluble coordination compounds and lipophilic drugs, usually resulting in moderate to high loading capacities, depending on surfactant composition and sonication energy [35].

Other antifungal nanoemulsions have shown comparable advantages. For instance, Garcia et al. (2019) [10] reported that the nanoemulsifier NB-201 exhibited robust activity against *C. albicans* in vitro and in vivo, attributed to the sustained exposure of the antifungal agent. Similarly, Pannu et al. (2009) [36] described NB-002 and other topical nanoemulsions

as formulations with superior antimicrobial efficacy compared to their free counterparts. Multiple studies have confirmed that silver nanomaterials exhibit potent antifungal activity against *C. albicans*, mainly by generating reactive oxygen species, disrupting membranes, and inhibiting biofilm formation [16,36,37]. However, this efficacy heavily depends on keeping silver soluble and bioavailable, a role that nanoemulsions effectively fulfill by preventing premature precipitation and controlling release kinetics. Recent reviews highlight that nanostructured delivery systems are promising strategies to overcome the inherent limitations of free silver compounds and to fight persistent fungal biofilms [38,39].

The images obtained through Transmission Electron Microscopy (TEM) showed that both the B-NL and Ag-NL formulations primarily have a spherical shape and consistent size distribution. The preservation of their shape after adding $[\text{Ag}(\text{PhTSC} \cdot \text{HCl})_2](\text{NO}_3) \cdot \text{H}_2\text{O}$ indicates that the lipid nanoemulsion effectively tolerated the encapsulation process, with no visible clumping or significant changes in their colloidal structure. These findings match the dynamic light scattering (DLS) data, which confirmed that both formulations have particle sizes below 200 nm. Such sizes are ideal for controlled drug delivery because they enhance tissue penetration and help evade early detection by cellular defense mechanisms [18–20].

TEM remains an essential complementary technique to DLS for assessing the morphology, homogeneity, and structural behavior of lipid nanoparticles. Sato et al. (2022) [40] demonstrated that lipid nanocarriers containing hypericin maintained their spherical shape and physicochemical stability in a vaginal gel system even after extended storage. Similarly, the present TEM analysis confirms that the nanostructured organization of the lipid system was preserved after silver complex loading, supporting the robustness of the formulation against the incorporation of bioactive metallic compounds.

Overall, our findings are in strong agreement with the literature, indicating that the incorporation of the silver complex into lipid-based nanostructured systems does not compromise morphology or stability, thereby preserving the structural and pharmaceutical properties essential for safe and effective application [41].

Rheological tests on the B-LN and Ag-LN formulations revealed non-Newtonian behavior, evidenced by the absence of a linear relationship between shear stress and shear rate, a common feature in systems containing stabilizing agents [42]. Oscillatory analysis showed predominantly elastic behavior, with the storage modulus (G') exceeding the loss modulus (G'') in both formulations ($G' = 449.84 \text{ Pa} > G'' = 34.69 \text{ Pa}$ for B-NL; $G' = 978 \text{ Pa} > G'' = 121.5 \text{ Pa}$ for Ag-LN). According to Hu, polymer-stabilized emulsions form interconnected structural networks that enhance elasticity and resistance to flow [43].

These findings match previous studies, highlighting the importance of a balanced formulation in shaping the viscoelastic and thixotropic traits of nanoemulsions. The interaction among components is essential for creating stable and effective nanostructured emulsions, aiding progress in controlled drug delivery systems.

In antimicrobial assessments, both global incidence rates and epidemiological trends of fungal infections are crucial for guiding prevention, diagnosis, and treatment strategies. The increasing prevalence of fungal infections is primarily driven by the rising number of immunocompromised individuals, such as those with HIV, cancer patients, transplant recipients, and elderly populations. This situation underscores the urgent need for new antifungal therapies, as traditional treatments often have limited effectiveness and are facing growing resistance. Coordination compounds have emerged as promising alternatives, demonstrating potential as effective antimicrobial agents [1,7,44].

The data from this study show that the silver coordination compound $[\text{Ag}(\text{PhTSC} \cdot \text{HCl})_2](\text{NO}_3) \cdot \text{H}_2\text{O}$ exhibits strong antifungal activity, especially against *Candida albicans*. It has better performance compared to standard agents like fluconazole, which was ineffective against some strains, highlighting its potential as an alternative treatment. When incorpo-

rated into the lipid nanoemulsion (Ag-LN), the antifungal activity was maintained, with MIC and MFC values similar to those of the free compound. This suggests that the lipid formulation preserves effectiveness while providing additional advantages, such as improved stability, better biocompatibility, and the possibility of controlled drug release [9,45,46].

Evaluation of the complex components, silver nitrate (AgNO_3) and the thiosemicarbazone ligand (TSC), revealed inherent antifungal activity; however, the coordinated complex showed significantly enhanced effectiveness. This enhancement likely results from synergistic interactions between the components and the increased molecular stability provided by metal coordination. Literature reports support these findings. For instance, Savić et al. evaluated several Ag(I) coordination compounds containing 1,7-phenanthroline ligands against multiple *Candida* strains. They observed notable antifungal activity, reduction in virulence, and reactive oxygen species (ROS)-mediated effects targeting fungal DNA [47].

These findings collectively show the effectiveness of adding the silver (I) coordination compound into lipid-based nanostructured systems. The developed formulations kept their physicochemical stability, had sustained release, and maintained antifungal activity, supporting their potential as promising options for treating *Candida albicans* infections.

To complement the biological evaluation, the mutagenic potential of the developed nanoformulation was also assessed using the Ames test, to ensure its toxicological safety and suitability for biomedical applications.

The results obtained demonstrate that Ag-NL did not induce point mutations in any of the *Salmonella* strains tested, regardless of metabolic activation. The absence of significant differences between the treated groups and the negative control, combined with an MI < 2.0, confirms the lack of mutagenic potential according to the criteria established by OECD 471.

Similar findings have been described for silver-based nanostructured systems, which generally exhibit low genotoxicity due to the encapsulation of Ag^+ ions within the lipid matrix, limiting direct interaction with bacterial DNA. The presence of phospholipids and surfactants, such as Brij[®] 35, may also contribute to the system's biocompatibility by mitigating oxidative stress and ionic release.

Overall, the data obtained support the toxicological safety of the developed formulation, indicating that the nanoemulsion acts as a protective carrier system for the coordination complex, preventing mutagenic effects even at the highest concentrations tested.

To further assess the safety profile of the developed nanoformulation, toxicity tests were conducted using *Galleria mellonella* larvae, a validated alternative model for pre-clinical toxicity studies.

Various tests are used to evaluate the toxic potential of substances, whether natural or synthetic. Traditionally, in vivo vertebrate models are employed to assess pathogen virulence, antimicrobial effectiveness, and toxicity profiles of new formulations. However, *Galleria mellonella* larvae have become a popular, ethical, and cost-effective alternative for testing the toxic potential of drugs and antimicrobial agents, including antifungal compounds [48]. One of the main advantages of this model is its high level of functional and structural similarity to the innate immune system of vertebrates, which includes both cellular and humoral defense mechanisms mediated by phagocytic hemocytes and the production of antimicrobial peptides similar to defensins [48,49].

The results of this study show that the free silver compound was significantly more toxic, with noticeable drops in larval survival rates—especially at doses of 200 and 250 mg/kg—indicating a clear dose-dependent trend. In contrast, the Ag-NL formulation lessened these effects, maintaining more stable survival curves and lower mortality even at higher doses. This suggests that nanoencapsulation of the compound enables controlled release, reducing toxicity by managing bioavailability and limiting direct interaction

with biological structures. Similar results were reported by Gandra et al. [50], who found variable toxicity among copper(II), manganese(II), and silver (I) chelates in *G. mellonella*, highlighting how the type and concentration of metal ions influence the toxic response.

The comparison between the free compound adding $[\text{Ag}(\text{PhTSC}\cdot\text{HCl})_2](\text{NO}_3)\cdot\text{H}_2\text{O}$ and its nanostructured formulation (Ag-NL) supports these observations: while the free compound caused significant mortality after 72 h, the nanostructured version showed reduced toxicity, evidenced by stable survival rates at the same concentrations. These results confirm that nanoencapsulation is an effective strategy to reduce acute toxicity by slowing the release of the active compound and avoiding systemic concentration peaks.

The lipid matrix of the nanoemulsion may also offer physical protection to the silver complex, reducing its direct interaction with cellular membranes. These protective effects align with studies showing that lipid nanoemulsions improve antifungal effectiveness while decreasing the toxicity of bioactive compounds, even against resistant *Candida albicans* strains [51,52].

The *Galleria mellonella* model has been extensively validated for the pre-clinical evaluation of nanoparticle toxicity, showing good correlation with vertebrate models and particular sensitivity for detecting acute toxic effects. In the case of silver nanoparticles, toxicity is commonly associated with Ag^+ ion release, leading to oxidative stress, disruption of enzymatic activity, and damage to cellular membranes [53,54].

Complementary studies on *Candida albicans* confirm the strong antifungal effect of silver-based nanomaterials, including preventing biofilm formation, disrupting membranes, and working well with standard antifungal drugs [9,45,46]. Therefore, the current data support that adding $[\text{Ag}(\text{PhTSC}\cdot\text{HCl})_2](\text{NO}_3)\cdot\text{H}_2\text{O}$ into a lipid nanoemulsion is an effective way to reduce the toxicity linked to the free compound, without losing its antifungal effectiveness, especially against *Candida albicans*, a clinically important pathogen because of its ability to form biofilms and its natural multidrug resistance.

4. Materials and Methods

4.1. Preparation of the Ligand (PhTSC) and the Silver (I) Complex $[\text{Ag}(\text{PhTSC}\cdot\text{HCl})_2](\text{NO}_3)\cdot\text{H}_2\text{O}$

The ligand 2-pyridinecarboxaldehyde-N(4)-phenylthiosemicarbazone hydrochloride (PCAPhTSC·HCl) was synthesized following the procedure reported [22]. The silver (I) coordination compound $[\text{Ag}(\text{PhTSC}\cdot\text{HCl})_2](\text{NO}_3)\cdot\text{H}_2\text{O}$ was obtained by dissolving 242.4 mg (0.78 mmol) of PCAPhTSC·HCl in acetonitrile and adding 66.3 mg (0.39 mmol) of AgNO_3 in a 2:1 molar ratio. The mixture was stirred for 4 h at room temperature ($26 \pm 1^\circ\text{C}$), affording a yellow precipitate. The solid was isolated by filtration, washed with diethyl ether, and dried under vacuum, yielding 80%. The compound was characterized by FT-IR, ^1H NMR, and ^{13}C NMR spectroscopy, in agreement with previous reports [22]. A schematic representation of the synthetic procedure is shown in Figure 10.

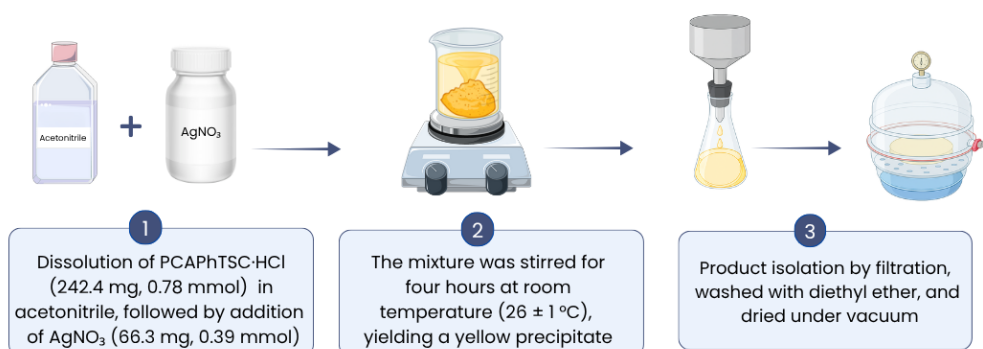


Figure 10. Scheme of the synthetic procedure for the silver (I) coordination compound $[\text{Ag}(\text{PhTSC}\cdot\text{HCl})_2](\text{NO}_3)\cdot\text{H}_2\text{O}$.

4.2. Nanostructured Lipid System Preparation

Lipid nanoemulsions (NLs) were prepared according to the method described by [55] with modifications (Figure 11). The blank nanoemulsion (B-NL) was composed of 80% (*v/v*) aqueous phase, 10% (*v/v*) oil phase, and 10% (*v/v*) surfactant. The aqueous phase consisted of phosphate-buffered saline (PBS, 50 mmol·L⁻¹, pH 7.4), prepared with ultrapure water obtained from a Milli-Q purification system (Millipore, USA). The oil phase was composed of cholesterol (CHO, ≥99%, Sigma-Aldrich, St. Louis, MO, USA) and grape seed oil (GSO, pharmaceutical grade, Sigma-Aldrich, USA) in a 1:1 (*w/w*) ratio. The surfactant mixture was prepared with soybean phosphatidylcholine (SPC, ≥95%, Lipoid GmbH, Ludwigshafen, Germany) and polyoxyethylene (23) lauryl ether (Brij® 35, ≥99%, Sigma-Aldrich, USA) in a 1:2 (*w/w*) ratio to optimize interfacial stabilization and provide steric protection to the droplets. For preparation, the oil phase (CHO/GSO) was poured into the aqueous phase (PBS) under magnetic stirring for 10 min, resulting in a pre-emulsion. This dispersion was subsequently subjected to probe sonication (Q500, QSonica, Newtown, CT, USA) at 20% amplitude, in 30 s on/off cycles, for 12 min, while keeping the sample in an ice bath (4–8 °C). The resulting blank nanoemulsion (B-NL) was centrifuged at 11,180× *g* for 15 min to remove possible titanium residues released from the probe tip. To obtain the [Ag(PhTSC·HCl)₂](NO₃)·H₂O, it was incorporated into the pre-formed B-NL to the final concentration of 1000 µg·mL⁻¹ (≈1.29 mM; ≈1293 µM), followed by an additional continuous sonication step of 4 min under the same cooling conditions. Thus, a lipid nanoemulsion carrying the silver complex (Ag-LN) was obtained.

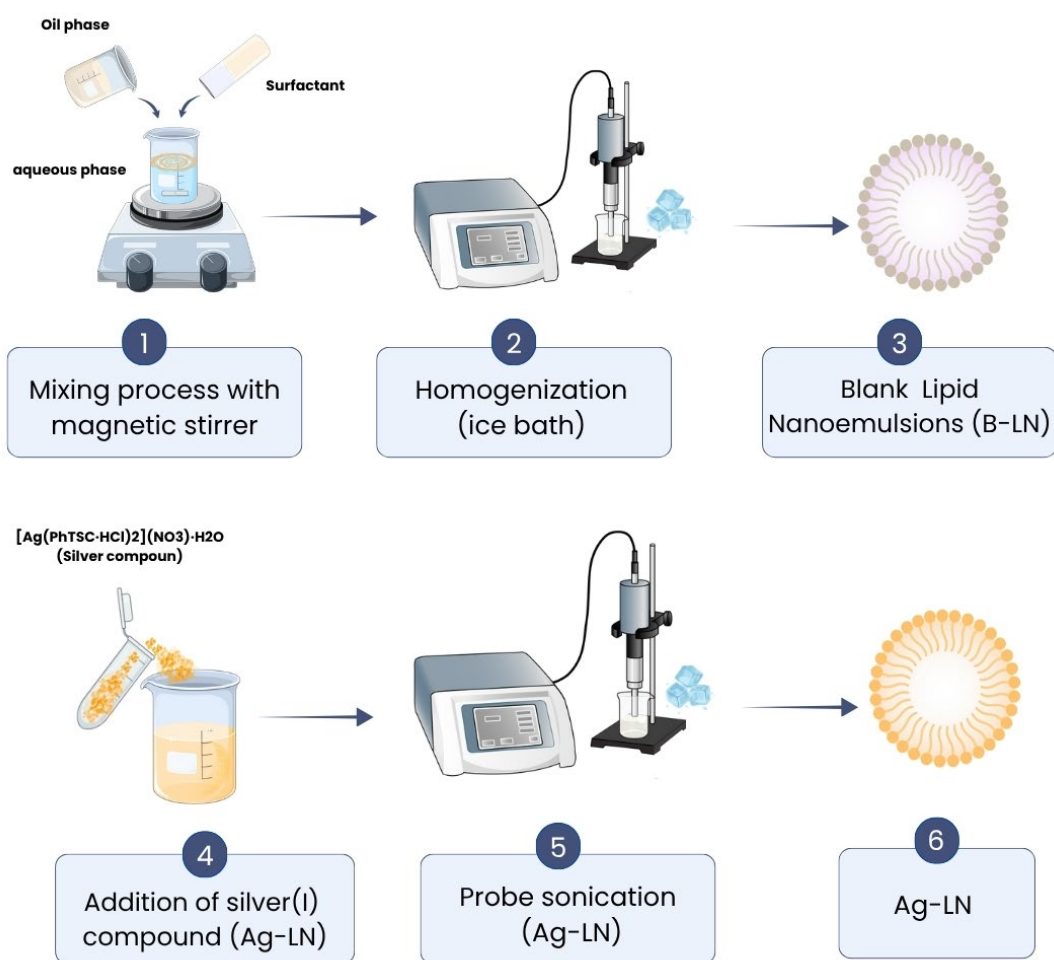


Figure 11. Schematic representation of the preparation of blank lipid nanoemulsion (B-LN) and silver-loaded lipid nanoemulsion (Ag-LN).

The B-LN was obtained by mixing the aqueous, oil, and surfactant phases under magnetic stirring, followed by probe sonication in an ice bath. The silver (I) complex $[\text{Ag}(\text{PhTSC} \cdot \text{HCl})_2](\text{NO}_3) \cdot \text{H}_2\text{O}$ was then incorporated by an additional sonication step, yielding the Ag-LN formulation.

4.3. Physical-Chemical Characterization of LNs

4.3.1. Size and Polydispersity Index (PDI)

The determination of particle size and diameter was conducted using a modified version of the technique proposed by [56]. Aliquots of B-NL and Ag-LN (10 μL diluted in 990 μL of ultrapure water at 25 $^\circ\text{C}$) were analyzed using photon correlation spectroscopy (Malvern Instruments, Malvern, Worcestershire, UK). The hydrodynamic diameter and polydispersity index (PDI) of the colloidal particles were calculated based on the Stokes–Einstein equation. Measurements were performed at a scattering angle of 173 $^\circ$ (backscatter) using a DTS1070 capillary electrophoresis cell (Malvern Instruments), composed of polycarbonate with integrated gold-plated copper electrodes. This configuration ensures chemical inertness and enables the analysis of a wide range of samples without electrode degradation.

4.3.2. Zeta Potential (ZP)

The zeta potential (ZP) of the nanoparticles was measured by electrophoretic mobility under the same conditions described above for particle size analysis. The surface potential of the formulations was calculated using the Smoluchowski equation [56].

4.3.3. Stability of B-NL and Ag-LN

We assessed the stability of B-NL and Ag-LN over 60 days. After preparation, we transferred aliquots of the formulations to sterile microtubes (Eppendorf, Hamburg, Germany) and stored them at approximately 4 $^\circ\text{C}$ under refrigeration, protected from light. Throughout this period, we measured the mean particle size, polydispersity index (PDI), and zeta potential (ZP) at set intervals (24 h, 7, 15, 30, 45, and 60 days).

4.4. In Vitro Drug Release

We conducted in vitro release assays under sink conditions using modified Franz diffusion cells (diffusion area 1.77 cm^2) in a Microette apparatus (Hanson Research, Chatsworth, CA, USA) with a cellulose membrane (Sigma-Aldrich, St. Louis, MO, USA). The receptor compartment was filled with 7.0 mL of phosphate buffer (pH 6.8). We continuously stirred the receptor medium at 300 rpm and kept the temperature at 37 ± 2 $^\circ\text{C}$ with a thermostatic water bath. The amount of silver compound released from the nanoemulsions was measured at 5, 15, 30, 45, 60, 90, 120, 210, 240, and 360 min. Silver concentration was determined by inductively coupled plasma mass spectrometry (ICP-MS, iCAP Qnova Series, Thermo Fisher Scientific, Waltham, MA, USA). Samples were diluted appropriately before analysis, and all measurements were performed in triplicate [57–59].

4.5. Transmission Electron Microscopy (TEM)

The samples were first diluted in PBS buffer (pH 7.4) and placed on a 200-mesh copper grid, allowing complete adsorption. Excess liquid was removed with filter paper. For negative staining, a drop of 1% (*w/v*) uranyl acetate solution was applied. After about 2 min, excess stain was removed, and the sample was air-dried at room temperature. Imaging was performed using a transmission electron microscope (JEOL JEM-100CXII) operating at an accelerating voltage of 200 kV and a magnification of 100,000 $\times$ [60].

4.6. Rheological Assays

Determination of Flow Properties

The LNB and LN-Ag formulations were analyzed using a controlled-stress rheometer (AR2000ex, TA Instruments, New Castle, DE, USA), maintained at 37 ± 0.1 °C. Depending on sample consistency, either cone/plate or plate/plate geometry was used; for standard analyses, cone/plate geometry was employed (40 mm diameter, 2° angle, 52 µm gap).

Small aliquots of each formulation were carefully placed on the lower plate of the rheometer to reduce shear stress during sample application. The samples sat for 3 min to ensure thermal equilibration. Flow properties were measured using a controlled shear rate test, where the shear rate was increased from 0 to 100 s^{-1} (ascending curve) and then decreased from 100 to 0 s^{-1} (descending curve). Each step lasted 120 s, with a 10 s pause between curves.

Oscillatory measurements were performed to evaluate the viscoelastic properties of the formulations. Initially, an amplitude sweep test was conducted at a constant frequency of 1 rad/s, with shear stress ranging from 0 to 50 Pa, to determine the linear viscoelastic region (LVR).

Based on this assay, a constant shear stress of 1 Pa was chosen for the frequency sweep test, which was performed over a frequency range of 0–10 Hz. From these analyses, the storage modulus (G') and the loss modulus (G'') were calculated, providing insight into the elastic and viscous behavior of the formulations [61].

4.7. Determination of Minimum Inhibitory Concentration (MIC_{90}) and Minimum Fungicidal Concentration (MFC) Against Candida Strains

4.7.1. Preparation of Microbial Suspensions

Five Candida strains were used in this study, obtained from the American Type Culture Collection (ATCC): *Candida albicans* (ATCC 10231), *Pichia kudriavzevii* (ATCC 6258), *Candida glabrata* (ATCC 2001), *Candida parapsilosis* (ATCC 22019), and *Candida tropicalis* (ATCC 750).

Yeast cultures grown for 48 h were transferred to Sabouraud dextrose broth (SDB) and adjusted to a 0.5 McFarland standard (approximately 10^6 CFU/mL). Cell density was confirmed by spectrophotometry at 530 nm and by manual counting using a Neubauer chamber. Subsequently, a 1:1000 dilution was performed, yielding a final concentration of approximately 10^3 CFU/mL.

4.7.2. Evaluation of Minimum Inhibitory Concentration (MIC_{90}) and Minimum Fungicidal Concentration (MFC) Against Candida Strains

The antifungal activity of $[\text{Ag}(\text{PhTSC} \cdot \text{HCl})_2](\text{NO}_3) \cdot \text{H}_2\text{O}$, LNB (blank nanoemulsion), Ag-LN (a coordination compound in lipid nanoemulsion), AgNO_3 , and TSC (thiosemicarbazone ligand) was assessed by measuring MIC_{90} using the broth microdilution method in 96-well plates, following the CLSI protocol (CLSI, 2017) with modifications. In each well, 100 µL of RPMI medium was added, followed by 100 µL of the test samples at concentrations ranging from 0.48 to 250 µg/mL [62].

For each assay, the following controls were included: growth control (RPMI + *C. albicans*), sterility control (sterile RPMI), solvent control (RPMI + 5% DMSO), and positive controls using amphotericin B (0.125–32 µg/mL) and fluconazole (0.5–128 µg/mL).

MIC_{90} was defined as the lowest concentration that inhibits $\geq 90\%$ of fungal growth compared to the growth control. Plates were incubated at 37 °C for 48 h. After incubation, 20 µL of a 2% aqueous solution of 2,3,5-triphenyltetrazolium chloride (TTC) was added to each well, and the plates were further incubated at 37 °C for 2 h. Absorbance was measured spectrophotometrically [63].

To determine the minimum fungicidal concentration (MFC), aliquots from wells showing no visible growth were plated onto Sabouraud Dextrose Agar (SDA) and incubated

at 37 °C for 48 h. Growth was assessed as described by [64], with modifications. For Ag-LN, the tested concentrations (0.48–250 µg/mL) were expressed as the equivalent amount of the encapsulated silver (I) coordination compound $[\text{Ag}(\text{PhTSC} \cdot \text{HCl})_2](\text{NO}_3) \cdot \text{H}_2\text{O}$, ensuring comparability with the free complex and AgNO_3 .

4.7.3. In Vitro Biofilm Formation and Inhibition Assay

The inhibition of biofilm formation was evaluated using *Candida albicans* in 96-well microplates, following the method of [64], with slight modifications. A fungal suspension of 5.0×10^8 cells/mL was prepared in sterile 0.9% saline and added to each well. The plates were incubated at 37 °C with gentle agitation (80 rpm) for 2 h to promote initial cell adhesion. After this period, wells were carefully washed with $[\text{Ag}(\text{PhTSC} \cdot \text{HCl})_2](\text{NO}_3) \cdot \text{H}_2\text{O}$ or Ag-LN at concentrations ranging from 15.6 to 2000 µg/mL, and then $[\text{Ag}(\text{PhTSC} \cdot \text{HCl})_2](\text{NO}_3) \cdot \text{H}_2\text{O}$ or Ag-LN was added. The plates were then incubated for another 24 h at 37 °C. Growth controls containing RPMI 1640 with *C. albicans* alone, sterility controls with RPMI only, solvent controls with RPMI plus 5% DMSO, and positive controls with amphotericin B (0.125–32 µg/mL) and fluconazole (0.5–128 µg/mL) were included. Biofilm metabolic activity was measured using the XTT reduction assay, with all conditions tested in triplicate and results expressed as the percentage of inhibition relative to the growth control [64,65].

4.7.4. In Vitro Mature Biofilm Inhibition Assay

Mature biofilms were evaluated using a similar initial adhesion procedure. A suspension of 5.0×10^8 cells/mL of *C. albicans* was added to 96-well microplates and incubated at 37 °C with gentle agitation (80 rpm) for 2 h. After adhesion, the supernatant was carefully removed, and 100 µL of RPMI 1640 medium was added to each well. The plates were then incubated at 37 °C for 24 h to allow biofilm development. Once mature biofilms formed, 100 µL of fresh RPMI medium and 100 µL of the test compounds (15.6–2000 µg/mL) were added, and the plates were incubated for an additional 48 h at 37 °C. Growth, sterility, solvent, and positive controls, identical to those used in the biofilm formation assay, were included. Metabolic activity was assessed using the XTT reduction assay, with all experiments performed in triplicate and results expressed relative to the growth control [64,65].

4.8. Mutagenicity Assessment (Ames Test)

The mutagenic potential of $[\text{Ag}(\text{PhTSC} \cdot \text{HCl})_2](\text{NO}_3) \cdot \text{H}_2\text{O}$ and Ag-LN was assessed using *Salmonella typhimurium* strains TA100 (detects base substitutions), TA98 (frameshift mutations), TA102 (mutations impacting AT base pairs), and TA97a (specific frameshift mutations). Tests were performed both with and without metabolic activation using the S9 liver microsomal fraction (Moltox Molecular Toxicology Inc., Boone, NC, USA), at concentrations selected from preliminary toxicity testing (0.62–5 µg/plate) [66,67].

Positive controls for assays with S9 included 2-anthramine (1.25 µg/plate) for TA97a, TA98, and TA100, and 2-aminofluorene (10 µg/plate) for TA102. For assays without S9, positive controls were 4-nitro-o-phenylenediamine (10 µg/plate) for TA97a and TA98, sodium azide (1.25 µg/plate) for TA100, and mitomycin C (0.5 µg/plate) for TA102. Negative controls included DMSO, spontaneous revertants, the blank nanoemulsion (B-NL), silver nitrate (AgNO_3), and thiosemicarbazone (TSC).

The mutagenicity index (MI) was calculated as the ratio of revertants per plate with the test compound to revertants per plate in the negative control. All experiments were performed in triplicate.

4.9. Acute Toxicity in *Galleria mellonella*

Acute toxicity was assessed using *Galleria mellonella* larvae, following [54], with minor adjustments. Eggs were incubated at 37 °C in darkness on an artificial diet made of honey,

glycerol, powdered milk, whole wheat flour, wheat germ, and wheat bran. Larvae weighing 200–320 mg and showing no signs of melanization were chosen for experiments (n = 10 per group) [54].

Test compounds were injected with a Hamilton 701N syringe with a 26 s needle (cone tip) delivering 10 µL into the last left proleg. Larvae were kept in Petri dishes in the dark and observed at 24, 48, and 72 h. The lack of response to physical stimuli, cuticle melanization, or complete loss of mobility defined mortality. Tested concentrations ranged from 5 to 250 mg/kg. Controls included needle-insertion (trauma), solvent (5% DMSO), and blank nanoemulsion (B-NL) controls. Ag-LN was tested at the highest dose (0.6 mg/kg) along with its base (B-NL). All experiments were conducted in triplicate.

4.10. Statistical Analysis

Statistical analyses were conducted using two-way analysis of variance (ANOVA), followed by Dunnett and Tukey post hoc tests, as well as Student's *t*-test. Data analysis was performed using GraphPad Prism 9.0 and Salanal 1.0 software, with linear regression used for analysis.

5. Conclusions

In this study, we successfully developed and characterized a lipid nanoemulsion incorporating the silver (I) coordination compound $[\text{Ag}(\text{PhTSC} \cdot \text{HCl})_2](\text{NO}_3) \cdot \text{H}_2\text{O}$ as an innovative nanostructured antifungal system. The formulation exhibited suitable physico-chemical features, maintaining colloidal stability and structural integrity while efficiently preserving the biological activity of the free complex.

Biological assays confirmed that nanoencapsulation not only maintained antifungal efficacy against *Candida* species but also enhanced biofilm inhibition and significantly reduced acute toxicity in *Galleria mellonella*. These findings demonstrate the dual advantage of lipid nanoemulsions as protective carriers and as active contributors to therapeutic performance, emphasizing their relevance for the treatment of biofilm-associated infections.

In addition to confirming its effectiveness, this work establishes a foundation for the translational development of Ag-LN as a next-generation antifungal platform. Future studies should focus on optimizing delivery routes, assessing long-term stability, and validating safety and efficacy in *in vivo* models. Altogether, this research strengthens the rationale for using silver-based nanocarriers as a versatile and promising approach to address the growing challenge of antifungal resistance.

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