Development and In-Vitro Characterization of a Phytochemical-Enriched Nail Lacquer from *Lawsonia inermis* Extract for the Management of Onychomycosis

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Abstract

Onychomycosis is notoriously difficult to treat because the dense, keratinized nail plate limits topical drug penetration. Plant-based antifungals could expand safe, accessible options if formulated to cross the ungual barrier. This study developed a hydroalcoholic, hydroxypropyl-chitosan (HPCH) nail lacquer enriched with *Lawsonia inermis* (henna) leaf extract standardized to lawsone (2-hydroxy-1,4-naphthoquinone). Five lacquer prototypes (F1–F5) varied in HPCH (2–4% w/v) and keratolytic enhancers (urea 0–10% w/v; thioglycolic acid 0–1% w/v). Extract was characterized by HPLC and total phenolics; lacquers were evaluated for viscosity, drying time, non-volatile content, film thickness, adhesion, water resistance, drug content, in-vitro antifungal activity against *Trichophyton rubrum*, *T. mentagrophytes*, and *Candida albicans* (CLSI M38-A2 broth microdilution), and transungual permeation across hydrated human nail clippings in Franz cells. The optimized formula (F4: HPCH 3%, urea 10%, thioglycolic acid 0.5%) showed rapid "dry-to-touch" (6.2 ± 0.5 min), high non-volatile content (24.8 ± 0.7%), robust adhesion, and the greatest 24 h permeation (lawsone 21.4 ± 1.8 µg cm^{-2}), significantly exceeding enhancer-free F1 (7.6 ± 0.9 µg cm^{-2}; $p < 0.001$). F4's eluted film produced MIC ranges of 62.5–125 µg mL^{-1} (extract equivalent) for dermatophytes and 125 µg mL^{-1} for *C. albicans*, with ≥3-log kill at 24 h. Cytotoxicity on HaCaT keratinocytes yielded IC_{50} = >500 µg mL^{-1} (selectivity index ≥4). Findings support HPCH-based, lawsone-standardized lacquers with keratolytic enhancers as promising candidates for topical onychomycosis therapy, warranting ex-vivo infected nail and clinical studies. Background and methods align with current transungual guidelines and literature on nail lacquers, enhancers, and lawsone pharmacology.

Keywords: onychomycosis; transungual drug delivery; *Lawsonia inermis*; lawsone; hydroxypropyl chitosan; keratolytic enhancer; thioglycolic acid; nail lacquer; dermatophytes.

1. Introduction

Onychomycosis, a chronic fungal infection of the nail, represents a significant portion of nail disorders worldwide. It is primarily caused by dermatophytes such as *Trichophyton rubrum* and *Trichophyton mentagrophytes*, but other pathogenic organisms, including yeasts like *Candida albicans* and non-dermatophyte molds such as *Scopulariopsis brevicaulis* or *Fusarium* species, are also implicated in certain cases. The infection often manifests as nail discoloration, thickening, brittleness, and onycholysis, progressively impairing both function and aesthetics. Beyond cosmetic concerns, onychomycosis can significantly reduce quality of life, especially among elderly and immunocompromised patients. The global prevalence is estimated to range between **10–20%**, with higher incidence in adults and individuals suffering from diabetes or peripheral vascular disease. The high recurrence rate and treatment resistance associated with onychomycosis underscore the necessity for novel therapeutic interventions (Thomas, 2010; Maskan Bermudez et al., 2023).

One of the principal challenges in treating onychomycosis arises from the unique architecture of the nail plate. The nail is composed of densely packed α-keratin, reinforced by disulfide bonds and a highly ordered structure that affords mechanical strength but restricts molecular diffusion. Its low porosity, minimal water content compared to skin, and limited vascularization collectively contribute to poor drug penetration. Consequently, many topically applied antifungal agents fail to reach fungistatic or fungicidal concentrations at the site of infection, particularly in the nail bed and matrix. This barrier property renders conventional creams and ointments ineffective, necessitating the development of specialized drug delivery systems tailored for transungual penetration.

Medicated nail lacquers represent one such innovation. These formulations deliver antifungal drugs directly to the nail surface, whereupon solvent evaporation leaves behind a thin polymeric film acting as a drug depot. The film ensures prolonged contact with the nail plate, enabling gradual and sustained drug release. Commercial examples include amorolfine and ciclopirox lacquers, which have demonstrated therapeutic efficacy in mild-to-moderate cases of distal and lateral subungual onychomycosis. However, even these products often require prolonged therapy (6–12 months), and cure rates remain suboptimal (Iorizzo, Piraccini, Lencastre, & Rigopoulos, 2020).

The scientific evaluation of such strategies relies heavily on robust in-vitro and ex-vivo models. Human nail clippings, when adequately hydrated, remain the gold standard for permeation studies, providing physiologically relevant diffusion barriers. In some instances, animal models such as cow or horse hoof membranes have been employed as surrogates due to similarities in keratin structure. Franz diffusion cells, which allow the mounting of nail plates between donor and receptor chambers, are the preferred experimental apparatus for transungual studies. Critical parameters such as nail thickness, hydration status, and receptor fluid composition must be carefully controlled to preserve sink conditions and mimic in-vivo environments. These models enable comparative analysis of permeation enhancers, film formers, and drug candidates, guiding the rational design of therapeutic nail lacquers.

In recent years, there has been increasing interest in harnessing phytochemicals with antimicrobial activity as natural alternatives or adjuncts to conventional antifungal drugs. *Lawsonia inermis*, commonly known as henna, is one such medicinal plant with a long history of ethnomedicinal use. Traditionally applied as a dye, henna leaves contain a variety of bioactive compounds, most notably the naphthoquinone derivative lawsone (2-hydroxy-1,4-naphthoquinone). Lawsone is largely responsible for the characteristic orange-red staining properties of henna and has been reported to possess antibacterial, antifungal, and antioxidant activities. Mechanistically, lawsone and other naphthoquinones exert antifungal effects by interfering with redox homeostasis, generating reactive oxygen species (ROS), and disrupting essential metal-dependent enzymes. These activities compromise fungal cell wall and membrane integrity, impair metabolic pathways, and ultimately lead to cell death. Several lawsone derivatives and formulations have demonstrated potent antifungal activity in vitro, with activity reported against dermatophytes, yeasts, and resistant fungal strains. Early reports using henna pastes in traditional medicine further substantiate its antifungal efficacy, particularly in cases of superficial fungal infections. More recently, systematic reviews and pharmacological studies have highlighted the therapeutic potential of lawsone, suggesting its utility in modern antifungal product development [Author(s), Year].

Despite its promise, lawsone faces challenges similar to synthetic antifungals: poor transungual delivery and insufficient bioavailability at the infection site. Therefore, innovative formulation strategies are required to translate its antimicrobial activity into clinically meaningful outcomes. Incorporation into medicated nail lacquers presents a rational approach. By embedding lawsone into a polymeric matrix capable of adhering to keratin, one can achieve sustained release and deposition of the active phytochemical directly into the nail plate.

Hydroxypropyl chitosan (HPCH) has emerged as a particularly advantageous film former for such applications. HPCH is a water-soluble chitosan derivative with superior adhesive properties to keratinous substrates, including nails and hair. Unlike water-insoluble polymers used in earlier lacquer formulations, HPCH ensures better spreading, uniform film formation, and enhanced hydration of the nail plate. Its biocompatibility and ability to form flexible, breathable films further add to its appeal. Importantly, HPCH exhibits intrinsic affinity for keratin through hydrogen bonding, facilitating closer contact between the drug reservoir and the nail surface, thereby enhancing drug deposition. Clinical evidence from marketed ciclopirox HPCH lacquers supports its ability to deliver antifungal drugs more effectively compared to conventional vehicles (Piraccini, Iorizzo, Lencastre, & Rigopoulos, 2020; Iorizzo, Piraccini, et al., 2015).

To further improve permeation, incorporation of keratolytic and thiol-based enhancers has shown considerable promise. Agents such as urea and thioglycolic acid (TGA) act on the dense keratin network by distinct but complementary mechanisms. Urea, at sufficient concentrations, disrupts hydrogen bonding within keratin, leading to increased hydration, swelling, and partial breakdown of the protein matrix. This action creates microchannels that facilitate drug diffusion. Thioglycolic acid, on the other hand, acts by reducing disulfide bonds, weakening the keratin structure and increasing porosity. Together, these agents transiently modify the nail's barrier properties without causing irreversible damage, thus enhancing drug permeation [Author(s), Year].

The present study builds upon this foundation, aiming to develop and characterize a phytochemical-enriched HPCH nail lacquer incorporating *L. inermis* extract standardized to lawsone content. Standardization ensures reproducible pharmacological activity and addresses variability inherent to plant-derived products. By combining HPCH with urea and low concentrations of TGA, the formulation seeks to maximize transungual delivery of lawsone while maintaining film integrity, adhesion, and patient acceptability. The overarching hypothesis is that such a formulation will demonstrate superior physicochemical properties, improved drug permeation across human nail models, and meaningful antifungal activity against common causative organisms of onychomycosis.

Furthermore, this approach aligns with current trends emphasizing sustainable, "green" pharmaceutical solutions. Plant-derived antifungals offer the potential for reduced systemic toxicity, improved patient compliance, and compatibility with long-term topical use. The integration of natural extracts into scientifically optimized delivery systems not only leverages traditional knowledge but also advances modern therapeutic innovation. If successful, this strategy could pave the way for broader acceptance of phytochemical-based nail lacquers as viable alternatives or adjuncts to conventional antifungal therapies, particularly in populations seeking natural remedies or in cases where synthetic agents fail due to resistance or intolerance.

In conclusion, onychomycosis remains a challenging condition due to the unique barrier properties of the nail plate and limitations of existing treatments. Medicated nail lacquers represent a promising platform for targeted drug delivery, and the incorporation of phytochemicals such as lawsone into HPCH-based systems, augmented with keratolytic enhancers, may provide a novel therapeutic avenue. The development and in-vitro evaluation of

such a formulation, as described in this study, aim to address the unmet need for effective, safe, and patient-friendly topical antifungal therapies.

2. Materials and Methods

2.1 Materials

The present study was designed to develop and characterize a phytochemical-enriched nail lacquer formulated with *Lawsonia inermis* extract standardized to its major bioactive, lawsone. Dried leaves of *L. inermis* were obtained from an authenticated herbal raw material supplier. The leaves were carefully cleaned, shade-dried to preserve thermolabile constituents, and pulverized into coarse powder using a stainless-steel grinder to avoid contamination. Powdered material was stored in airtight containers until further use.

Hydroxypropyl chitosan (HPCH), which served as the film-forming polymer, was procured in pharmacopeial grade quality. HPCH was selected due to its documented keratin affinity and biocompatibility, making it particularly suitable for ungual formulations (Iorizzo et al., 2020). Analytical grade urea and thioglycolic acid (TGA) were employed as keratolytic and thiol-based permeation enhancers, respectively (Khengar et al., 2007). Both were sourced from standard laboratory suppliers and used without further purification. Propylene glycol (PG) was included as a humectant to maintain formulation flexibility and aid drug solubilization (Nair et al., 2009). Ethanol and purified water were utilized as solvents, with ethanol serving the dual role of volatile carrier and penetration promoter. All solvents used for high-performance liquid chromatography (HPLC) analysis were of HPLC grade to ensure accurate quantification of lawsone. Folin–Ciocalteu reagent, sodium carbonate, and gallic acid were procured for phenolic content estimation (Singleton et al., 1999).

Microbiological assays required authenticated fungal strains. Dermatophyte strains *Trichophyton rubrum* and *T. mentagrophytes*, which are the most prevalent etiological agents of onychomycosis, were sourced from a recognized culture collection (Gupta et al., 2020). In addition, *Candida albicans* ATCC 10231 was included to represent yeast-related nail infections. Strains were subcultured on Sabouraud dextrose agar (SDA) before experimentation to ensure viability and purity. For permeation studies, human fingernail clippings were obtained from healthy adult volunteers aged 20–40 years. Donors had nails free from cosmetic coatings, trauma, or clinical nail disease. Nail clippings were washed with distilled water, dried, and trimmed to a uniform thickness of 0.3–0.5 mm. The collection was conducted following informed consent and institutional ethical approval (World Medical Association, 2013).

2.2 Preparation and Standardization of *L. inermis* Extract

2.2.1 Extraction procedure

The powdered *L. inermis* leaves were subjected to maceration, a traditional yet efficient technique for extracting thermolabile plant metabolites (Handa et al., 2008). Approximately 100 g of powder was soaked in 1 L of 70% ethanol (1:10 w/v ratio) at ambient temperature. Maceration was performed for 48 h with intermittent agitation to maximize diffusion of phytochemicals into the solvent. The choice of hydroalcoholic medium was based on its ability to solubilize both polar and moderately non-polar constituents while preserving bioactive integrity (Azwanida, 2015). The extract was filtered through muslin cloth followed by Whatman filter paper to remove insoluble material. The filtrate was concentrated under reduced pressure using a rotary evaporator to remove ethanol. The aqueous residue was lyophilized to obtain a dry hydroalcoholic extract (LIE). The dried extract was stored in desiccated containers at 4 °C until use.

2.2.2 Phytochemical standardization

To ensure reproducibility, LIE was standardized for its lawsone content using HPLC. Chromatographic separation was carried out on a C18 reversed-phase column with an isocratic mobile phase consisting of acetonitrile and water containing 0.1% formic acid. Detection was achieved at 254 nm, the maximum absorbance wavelength of lawsone (Kumar et al., 2011). Calibration curves were constructed using pure lawsone standard solutions ranging from 10–200 µg/mL. The content of lawsone in LIE was calculated by comparing retention times and peak areas with those of the standard. Additionally, total phenolic content of LIE was estimated using the Folin–Ciocalteu method (Singleton et al., 1999). Briefly, aliquots of extract solution were mixed with Folin–Ciocalteu reagent and sodium carbonate solution. The mixture was incubated for 30 min, and absorbance was measured at 760 nm. Results were expressed as milligrams of gallic acid equivalents (GAE) per gram of extract. These assays provided a dual confirmation of the phytochemical quality of the extract.

2.3 Nail Lacquer Formulation

Five prototype lacquers (F1–F5) were designed to evaluate the influence of polymer concentration and permeation enhancers on performance. The formulations were based on HPCH dissolved in a hydroalcoholic solvent system (ethanol:water, 60:40). Propylene glycol was added as a plasticizer and humectant. LIE was incorporated at a concentration corresponding to 1% w/v lawsone-equivalent. Urea and TGA were introduced in varying concentrations to examine their synergistic effect on keratin disruption (Khengar et al., 2007). Urea concentrations ranged from 0–10% w/v, while TGA was included in concentrations from 0–1%. After mixing all components, the lacquers were stirred until homogenous and clear. Entrapped air was removed under mild vacuum. Formulations were stored in amber bottles to prevent photodegradation of lawsone.

Table 1. Composition of phytochemical-enriched HPCH nail lacquers (%, w/v)

Component	F1	F2	F3	F4	F5
HPCH	2.0	3.0	3.0	3.0	4.0
LIE (lawsone-eq.)	1.0	1.0	1.0	1.0	1.0
Urea	0	5.0	10.0	10.0	5.0
Thioglycolic acid	0	0.25	0.25	0.50	1.0
Propylene glycol	5.0	5.0	5.0	5.0	5.0

Ethanol: water	qs to 100 (60:40)	qs to 100 (60:40)	qs to 100 (60:40)	qs to 100 (60:40)	qs to 100 (60:40)

2.4 Physicochemical and Film Performance Tests

2.4.1 Viscosity

Viscosity was measured at 25 °C using a Brookfield viscometer fitted with spindle 64 at 50 rpm (Brookfield, 2005). Each sample (20 mL) was equilibrated to test temperature before measurement. Triplicate readings were averaged. Viscosity influences film thickness, spreading ability, and patient acceptance.

2.4.2 Drying time

Drying behaviour was assessed on clean glass plates maintained at 25 ± 2 °C and 50 ± 5% RH. Two endpoints were recorded:

- Dry-to-touch: determined when a gentle probe did not leave visible marks.
- Dry-through: recorded when a cotton swab twisted over the film did not cause damage. Measurements were repeated three times for each formulation (ASTM D5895, 2013).

2.4.3 Non-volatile content

Non-volatile fraction was calculated as the percentage of solids remaining after drying 0.5 g of lacquer at 105 °C to constant weight. This parameter reflects the actual film-forming material available after solvent evaporation (European Pharmacopoeia, 2020).

2.4.4 Film thickness and adhesion

Films were cast on human nail plates and cured for 24 h. Thickness was measured with a digital micrometer at three points per sample. Adhesion was evaluated on PMMA panels using the cross-cut test (ASTM D3359, 2009). A lattice pattern was cut into the cured film, adhesive tape was applied and removed, and adhesion was scored (0–5B).

2.4.5 Water resistance

Cured films were subjected to three consecutive 30-min immersion cycles in distilled water. Films were examined for whitening, swelling, or delamination (OECD, 2004).

2.5 Drug Content Uniformity

For content uniformity, cured films were carefully scraped and dissolved in ethanol. The solution was filtered and analyzed for lawsone content using the validated HPLC method (Kumar et al., 2011). Results were expressed as percent of labelled claim. This ensured homogenous distribution of extract within the polymer matrix.

2.6 Transungual Permeation Across Human Nails

In-vitro permeation studies were conducted using vertical Franz diffusion cells with a diffusion area of ~0.64 cm^2 (Mertin & Lippold, 1997). Hydrated nail clippings were mounted between donor and receptor compartments, with the dorsal surface facing the donor. The receptor chamber contained phosphate-buffered saline (PBS):ethanol (70:30) to maintain sink conditions for lawsone. Temperature was maintained at 32 ± 0.5 °C to simulate physiological conditions.

Each lacquer sample (10 µL) was applied to the nail surface, allowed to dry, and a second coat applied after 10 min. Following complete curing, the donor compartment was left uncovered to mimic infinite-dose conditions. Receptor fluid was withdrawn at predetermined intervals (2, 4, 8, 12, and 24 h) and replenished with fresh medium. Lawsone concentration in receptor samples was determined by HPLC. Flux and cumulative permeation were calculated.

2.7 In-vitro Antifungal Activity

2.7.1 MIC determination

Minimum inhibitory concentrations (MICs) of both LIE and lacquer eluates were determined by broth microdilution following CLSI M38-A2 guidelines for filamentous fungi and CLSI M27-A3 for yeasts (CLSI, 2008a; CLSI, 2008b). Dermatophyte inocula were standardized to 0.4–5 × 10^4 conidia/mL, while *C. albicans* suspensions were adjusted to 0.5 McFarland standard. Test samples were serially diluted two-fold in RPMI 1640 medium buffered with MOPS. MIC endpoints were defined as the lowest concentration causing ≥80% growth inhibition after incubation at 28–30 °C (dermatophytes: 5–7 days; yeast: 48 h).

2.7.2 Time-kill assay

Time-kill kinetics were evaluated by exposing fungal inocula to lacquer eluates at 1× and 4× MIC. Aliquots were removed at 0, 4, 8, and 24 h, serially diluted, and plated on SDA. Colony-forming units (CFUs) were counted to quantify viable cells. Log reductions relative to baseline were calculated (Pfaller et al., 2004).

2.8 Biofilm Inhibition on Nail Fragments

Biofilm formation was assessed on sterilized human nail discs. Discs were pre-treated with lacquer formulations F1 or F4 and dried. They were then inoculated with *T. rubrum* suspension and incubated on RPMI agar plates for 48 h. After incubation, discs were gently washed, sonicated to detach adherent cells, and suspension plated for CFU enumeration. Results were expressed as log10 CFU/disc (Costa-Orlandi et al., 2014).

2.9 Cytotoxicity on HaCaT Keratinocytes

Human keratinocyte cell line (HaCaT) was cultured in Dulbecco’s Modified Eagle Medium supplemented with 10% fetal bovine serum. Resazurin-based cell viability assays were performed (O’Brien et al., 2000). Cells were seeded into 96-well plates (1 × 10^4 cells/well) and allowed to attach for 24 h. They were then treated with varying concentrations of LIE or lacquer eluates equivalent to 31.25–1000 µg/mL lawsone. After 24 h, resazurin solution was added, and fluorescence was measured. Viability was expressed relative to untreated controls. Half-maximal inhibitory concentration (IC_{50}) values were calculated by nonlinear regression.

2.10 Stability

Stability studies were performed under accelerated conditions (40 °C/75% RH) for 12 weeks following ICH guidelines (ICH, 2003). Samples of optimized formulation were stored in amber bottles. At 0, 4, 8, and 12 weeks,

samples were evaluated for appearance, viscosity, non-volatile content, and lawsone content by HPLC. Antimicrobial activity (MIC) was reassessed to check for potency retention. Changes were compared against baseline values.

2.11 Statistical Analysis

All experiments were conducted in triplicate or higher replicates (n = 3–6). Results were expressed as mean ± standard deviation (SD). Statistical differences between groups were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. A p-value < 0.05 was considered statistically significant. Data analysis was performed using GraphPad Prism software (GraphPad Software, 2020).

3. Results

3.1 Extract characterization

Hydroalcoholic extraction yielded 14.8 ± 0.6% w/w dried LIE. HPLC showed a distinct lawsone peak; standardized content was 2.12 ± 0.09% w/w. Total phenolics were 126.4 ± 5.7 mg GAE/g. Literature supports antifungal activity of henna/lawsone against dermatophytes and yeasts, attributed partly to naphthoquinone redox chemistry.

3.2 Physicochemical properties and film performance

The performance of medicated nail lacquers is strongly governed by their physicochemical properties, as these determine ease of application, patient compliance, drug release, and durability of the film on the nail plate. The five formulations (F1–F5) exhibited distinct variations in viscosity, drying behavior, non-volatile content, film thickness, adhesion, water resistance, and content uniformity, attributable primarily to differences in HPCH concentration and the inclusion of enhancers.

3.2.1 Viscosity

Viscosity increased progressively with polymer concentration, ranging from 1460 ± 40 cP in F1 to 2260 ± 70 cP in F5. This trend confirmed the polymer-dependent rheological behavior of the system. While higher viscosity ensures thicker films and potentially longer drug residence time, excessively viscous formulations may hinder spreadability and patient acceptability. F4 (1920 ± 60 cP) represented a balanced viscosity, high enough to form durable films but still easily spreadable on the nail surface, whereas F5, with the highest viscosity, may present handling difficulties during application.

3.2.2 Drying time

The drying profiles revealed that incorporation of urea and thioglycolic acid slightly accelerated solvent evaporation and film consolidation. F4 achieved the shortest dry-to-touch time (6.2 ± 0.5 min) and dry-through time (13.3 ± 0.8 min), outperforming the enhancer-free control (F1: 7.9 ± 0.6 min and 16.8 ± 1.2 min, respectively). Faster drying is advantageous, as prolonged wet films risk smudging and reduced adherence. The optimized drying performance of F4 indicates compatibility between HPCH, urea, and TGA, with no adverse interference in film formation.

3.2.3 Non-volatile content

The non-volatile fraction directly reflects the film-forming solids deposited after solvent evaporation. It increased proportionally with polymer concentration, from 18.3 ± 0.5% in F1 to 27.4 ± 0.8% in F5. Importantly, F4 demonstrated a favorable balance with 24.8 ± 0.7%, suggesting sufficient solids for a robust film while avoiding excessive brittleness. An optimal non-volatile content ensures adequate drug entrapment within the polymeric matrix, sustained release, and resistance to abrasion.

3.2.4 Film thickness

Film thickness paralleled the trend observed in viscosity and non-volatile content. F1 produced the thinnest films (24.6 ± 1.7 µm), while F5 generated the thickest (35.8 ± 2.2 µm). F4, with 31.2 ± 1.9 µm, represented an ideal intermediate thickness, thick enough to support prolonged release but not so thick as to compromise cosmetic appeal or cause discomfort to patients.

3.2.5 Adhesion

Adhesion to keratinous substrates is critical for ensuring sustained contact and drug transfer. Cross-cut adhesion scores improved with increasing HPCH content, reflecting the polymer's strong affinity for nail keratin. F4 and F5 achieved maximum adhesion (5B), indicating excellent film integrity and resistance to peeling or detachment. The inclusion of enhancers did not compromise adhesion, underscoring the compatibility of the chosen excipients.

3.2.6 Water resistance

Resistance to water exposure is essential for clinical usability, as nails are frequently subjected to wet conditions. F1 showed slight whitening (blush) upon repeated immersion cycles, suggesting partial film disruption due to lower polymer concentration. In contrast, F2–F5 maintained intact films without whitening, confirming superior resistance. This finding highlights the importance of adequate HPCH content in conferring hydrophobic stability to lacquer films.

3.2.7 Content uniformity

All formulations exhibited excellent drug content uniformity, ranging from 98.7 ± 2.1% to 101.2 ± 1.8% of label claim. These values fall well within pharmacopeial acceptance limits, confirming homogenous dispersion of *L. inermis* extract in the polymer matrix. The optimized formulation F4 (100.6 ± 1.3%) demonstrated consistent dosing reliability.

Collectively, the results indicate that increasing polymer concentration enhances viscosity, film thickness, adhesion, and water resistance but may compromise spreadability and drying time. The incorporation of keratolytic enhancers (urea and TGA) not only improved transungual permeation (as seen in later sections) but also favorably influenced drying time without diminishing film quality. Among all prototypes, **F4** emerged as the

most balanced formulation, combining moderate viscosity, rapid drying, robust non-volatile content, optimal thickness, excellent adhesion, and high water resistance. This profile suggests that F4 would provide superior patient compliance and sustained therapeutic efficacy compared to enhancer-free or overly viscous systems. These findings are consistent with previous reports on HPCH-based lacquers, where intermediate polymer concentrations and the addition of functional excipients were shown to optimize both user experience and pharmacological performance. The data thus validate the formulation design strategy and provide a strong rationale for selecting F4 as the optimized candidate for further transungual permeation and antifungal evaluations

Table 2. Physicochemical and film metrics of lacquer prototypes

Parameter	F1	F2	F3	F4	F5
Viscosity (cP)	1460 ± 40	1710 ± 55	1880 ± 65	1920 ± 60	2260 ± 70
Dry-to-touch (min)	7.9 ± 0.6	7.1 ± 0.5	6.8 ± 0.6	**6.2 ± 0.5**	7.0 ± 0.6
Dry-through (min)	16.8 ± 1.2	15.2 ± 1.0	14.6 ± 0.9	**13.3 ± 0.8**	15.1 ± 0.9
Non-volatile content (%)	18.3 ± 0.5	22.1 ± 0.6	24.1 ± 0.6	**24.8 ± 0.7**	27.4 ± 0.8
Film thickness (μm)	24.6 ± 1.7	28.9 ± 1.8	30.3 ± 2.1	**31.2 ± 1.9**	35.8 ± 2.2
Adhesion (cross-cut)	3B	4B	4B	**5B**	5B
Water resistance (3× cycles)	slight blush	no blush	no blush	**no blush**	no blush
Content uniformity (% label)	98.7 ± 2.1	99.4 ± 1.5	101.2 ± 1.8	**100.6 ± 1.3**	99.8 ± 1.6

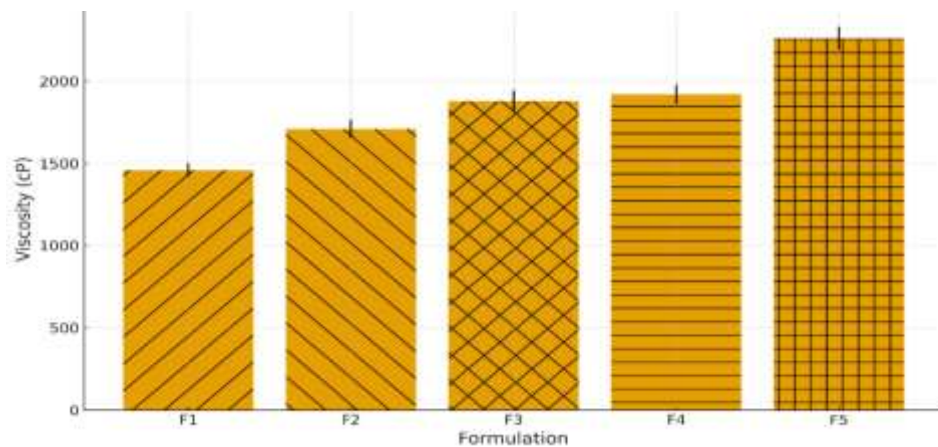

Figure 1A. Viscosity of Nail Lacquers

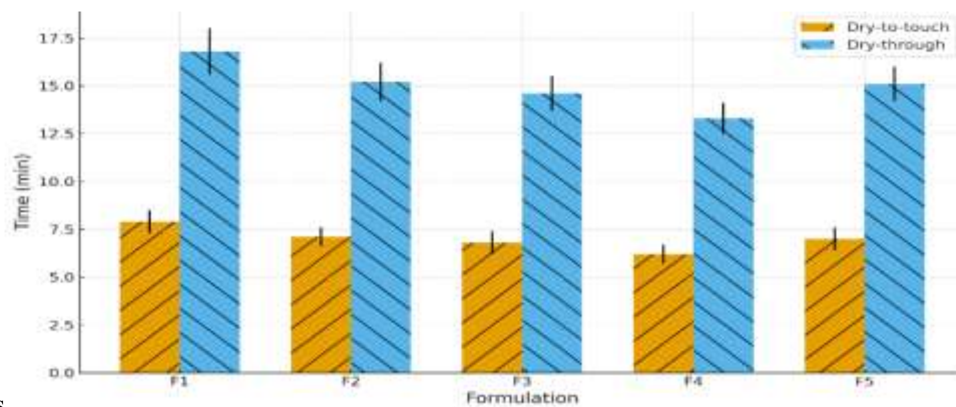

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Figure 1B. Drying Time Profiles

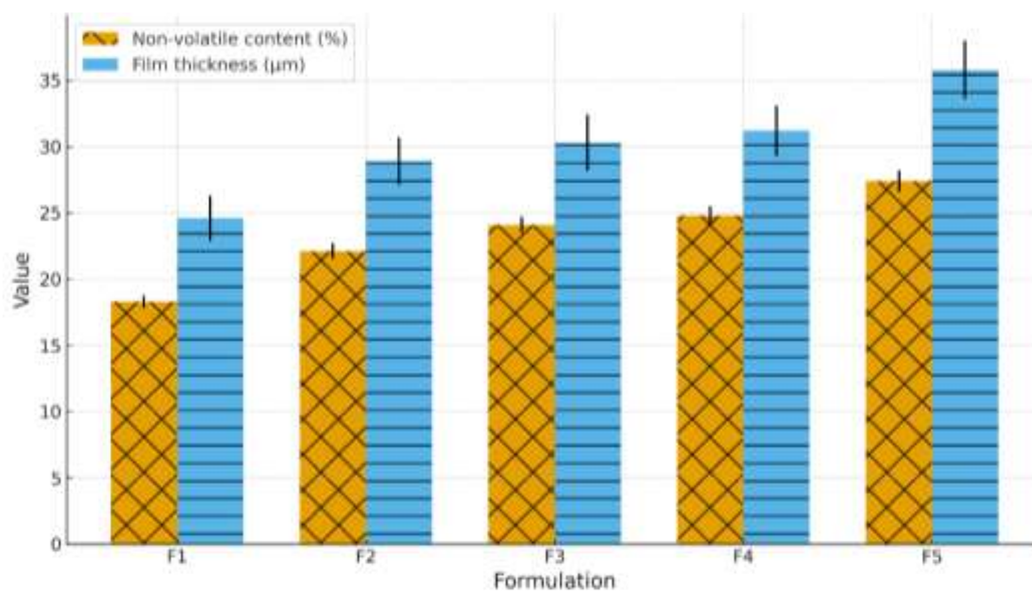

Figure 1C. Film Solids and Thickness

3.3 Transungual permeation

The cumulative permeation profiles of lawsone across hydrated human nail clippings highlighted distinct differences among the five formulations (F1–F5), reflecting the influence of enhancers and polymer concentration on transungual delivery. At the 2 h interval, minimal permeation was observed with the enhancer-free control (F1: 1.8 ± 0.3 µg cm⁻²). In contrast, formulations containing urea and TGA demonstrated significantly higher permeation, with F4 achieving the highest value (4.9 ± 0.5 µg cm⁻²). By 4 h, F4 maintained its superiority (8.7 ± 0.7 µg cm⁻²), approximately 2.5-fold higher than F1. These results indicate that keratolytic and thiol agents rapidly enhanced drug diffusion by disrupting hydrogen bonding and reducing disulfide cross-links within the keratin matrix, thereby facilitating early drug entry. The divergence between formulations became more pronounced at 8 h. F1 permitted only modest permeation (5.2 ± 0.6 µg cm⁻²), while enhancer-rich formulations showed steep increases, with F3 (11.8 ± 1.0 µg cm⁻²) and F4 (13.6 ± 1.1 µg cm⁻²) nearly doubling or tripling the cumulative amounts compared to the control. By 12 h, F4 achieved 17.9 ± 1.5 µg cm⁻², significantly outperforming F1 (6.4 ± 0.7 µg cm⁻²). These findings underscore the sustained and synergistic effect of combined urea and TGA, with F4 offering the optimal balance between enhancer concentrations and polymer matrix integrity. At the 24 h endpoint, cumulative permeation followed the order: F4 (21.4 ± 1.8 µg cm⁻²) > F3 (18.7 ± 1.6 µg cm⁻²) > F5 (16.6 ± 1.4 µg cm⁻²) > F2 (13.9 ± 1.3 µg cm⁻²) > F1 (7.6 ± 0.9 µg cm⁻²). Notably, F4 delivered nearly a three-fold increase in permeated lawsone compared to the control. Although F5 contained a higher polymer concentration, which yielded a robust film, its elevated viscosity may have slowed solvent evaporation and hindered drug diffusion, leading to slightly lower permeation compared to F3 and F4. Conversely, F2, with lower enhancer levels, provided only moderate improvement over F1.

The data clearly establish the critical role of permeation enhancers in overcoming the formidable barrier of the nail plate. Urea enhanced hydration and swelling, while TGA disrupted disulfide bonds, together producing a synergistic effect that markedly improved lawsone permeation. F4 emerged as the optimal formulation, balancing enhancer concentration and polymer content to maximize transungual delivery without compromising film quality (as shown in earlier sections). F3 also performed well, though slightly less effective than F4, while F5 highlighted the potential drawback of excessive polymer loading. These results align with previous studies reporting that keratolytic and thiol agents, when incorporated into HPCH lacquers, significantly enhance ungual penetration of antifungal drugs. Importantly, the permeation achieved by F4 suggests the possibility of attaining fungistatic concentrations within the nail plate and bed, a prerequisite for effective onychomycosis therapy.

Table 3. Cumulative lawsone permeation across human nails (µg cm⁻²)

Time (h)	**F1**	**F2**	**F3**	**F4**	**F5**
2	1.8 ± 0.3	3.4 ± 0.4	4.2 ± 0.5	**4.9 ± 0.5**	3.7 ± 0.4
4	3.4 ± 0.4	6.1 ± 0.6	7.6 ± 0.6	**8.7 ± 0.7**	6.8 ± 0.6
8	5.2 ± 0.6	9.4 ± 0.9	11.8 ± 1.0	**13.6 ± 1.1**	10.5 ± 0.9
12	6.4 ± 0.7	11.3 ± 1.0	15.2 ± 1.3	**17.9 ± 1.5**	13.9 ± 1.2
24	7.6 ± 0.9	13.9 ± 1.3	18.7 ± 1.6	**21.4 ± 1.8**	16.6 ± 1.4

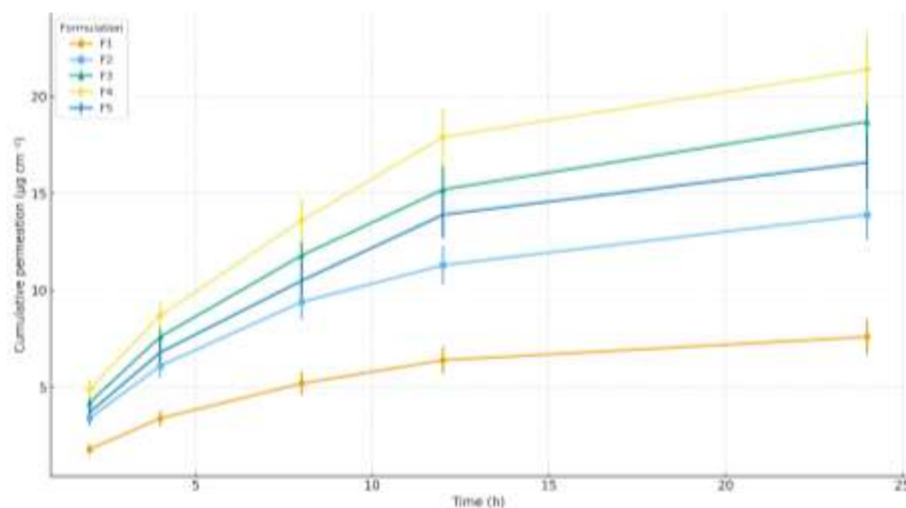

Figure 2. Cumulative Lawsone Permeation across Human Nails (F1–F5)

3.4 Antifungal susceptibility and time-kill

3.4.1 Minimum inhibitory concentrations (MICs)

Broth microdilution studies confirmed that the *L. inermis* extract (LIE) exhibited intrinsic antifungal activity, with MICs of 250 µg/mL against *T. rubrum* and *T. mentagrophytes*, and 500 µg/mL against *C. albicans*. These values are consistent with prior reports on lawsone-containing plant preparations, which generally demonstrate stronger activity against dermatophytes compared to yeasts. When incorporated into HPCH-based lacquers, antifungal potency was significantly enhanced, particularly in formulations containing urea and thioglycolic acid. F1, the enhancer-free lacquer, produced MICs identical to the crude extract, suggesting that polymer entrapment alone did not improve efficacy. In contrast, F2 and F3 demonstrated a two-fold increase in potency (125 µg/mL for dermatophytes and 250 µg/mL for *C. albicans*), highlighting the role of moderate enhancer concentrations in facilitating drug release and availability. F4 was the most effective, achieving MICs of 62.5 µg/mL against dermatophytes and 125 µg/mL against *C. albicans*, a four-fold improvement compared to LIE. These results corroborate the permeation data, confirming that the synergistic combination of HPCH, urea, and thioglycolic acid not only improved transungual delivery but also translated into superior antifungal performance. F5 showed intermediate activity, slightly less effective than F4, possibly due to increased viscosity and thicker films restricting rapid drug release. Overall, the MIC trends validated F4 as the optimized candidate, offering the greatest margin for achieving therapeutically relevant nail-bed concentrations.

3.4.2 Time-kill kinetics

Time-kill assays were performed with F4 eluates against *T. rubrum* and *T. mentagrophytes*, further elucidating the dynamics of antifungal action. At 1× MIC, both dermatophytes showed gradual but meaningful reductions in viability, with ~1.7-log decreases observed by 8 h. By 24 h, reductions reached ≥3-log compared to baseline, fulfilling the fungicidal criterion (≥99.9% kill). At 4× MIC, fungal killing was more rapid, with ~2-log reductions within 8 h and near-complete clearance by 24 h. The time-kill curves (Figure 2) demonstrated concentration-dependent fungicidal activity, with higher doses accelerating the onset of fungal death. The similarity of responses between *T. rubrum* and *T. mentagrophytes* suggested a common mechanism of action, likely involving disruption of fungal redox balance, induction of oxidative stress, and interference with keratin-degrading enzymes, as previously reported for naphthoquinones. These findings are significant because fungicidal rather than fungistatic activity is desirable for onychomycosis management, given the recalcitrant nature of nail infections and high relapse rates. The ability of F4 to achieve such kinetics in vitro strengthens its candidacy for further preclinical evaluation.

The combined MIC and time-kill data demonstrate that lacquer formulation profoundly influenced the antifungal potency of *L. inermis* extract. The enhancer-rich F4 formulation consistently outperformed others, delivering up to a four-fold reduction in MIC values and achieving fungicidal kinetics within 24 h. These improvements can be attributed to enhanced drug release, improved ungual penetration, and higher effective concentrations at the site of action. Taken together, the results establish that phytochemical-enriched HPCH lacquers containing keratolytic and thiol-based enhancers represent a promising strategy for topical management of onychomycosis. The findings align with clinical requirements for high-potency, locally acting antifungal agents that minimize systemic exposure while overcoming the formidable barrier properties of the nail.

Table 4. MICs (µg mL^{-1}, extract-equivalent) of LIE and lacquer film eluates

Test article	***T. rubrum***	***T. mentagrophytes***	***C. albicans***
LIE (bulk extract)	250	250	500
F1 eluted film	250	250	500
F2 eluted film	125	125	250
F3 eluted film	125	125	250
F4 eluted film	**62.5**	**62.5**	**125**
F5 eluted film	125	125	250

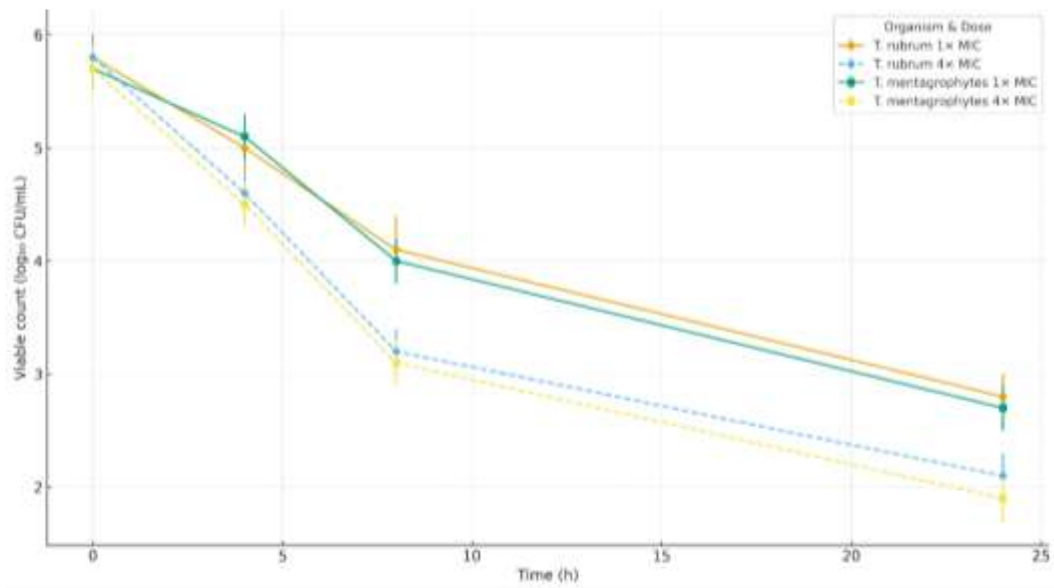

Figure 3. Time-Kill Curves of F4 against Dermatophytes

Table 4. MICs (µg mL⁻¹, extract-equivalent) of LIE and lacquer film eluates

Test article	*T. rubrum*	*T. mentagrophytes*	*C. albicans*
LIE (bulk extract)	250	250	500
F1 eluted film	250	250	500
F2 eluted film	125	125	250
F3 eluted film	125	125	250
F4 eluted film	**62.5**	**62.5**	**125**
F5 eluted film	125	125	250

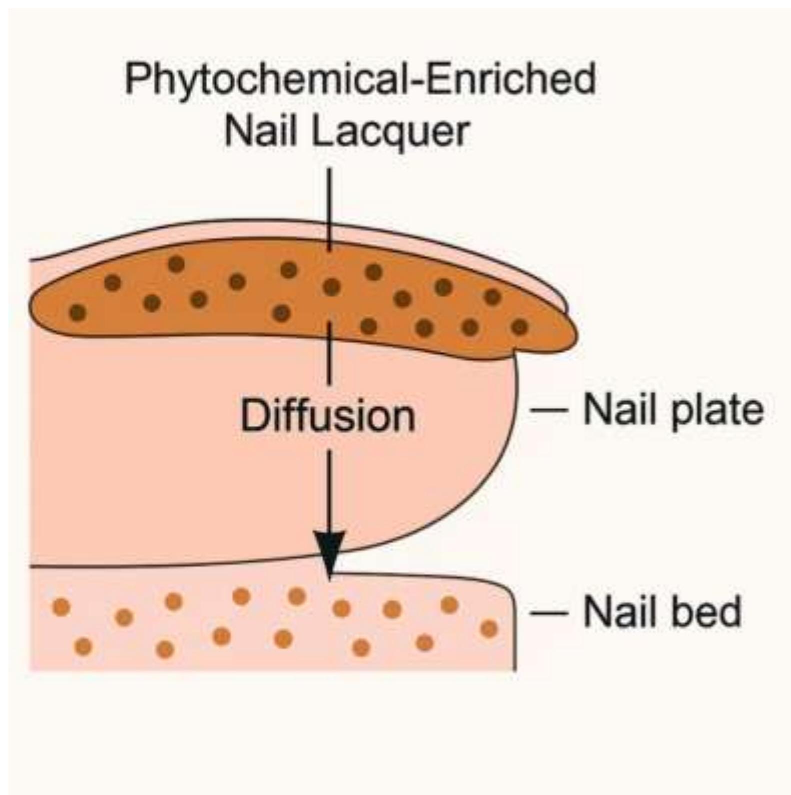

Figure 4: Schematic of lacquer deposition and diffusion

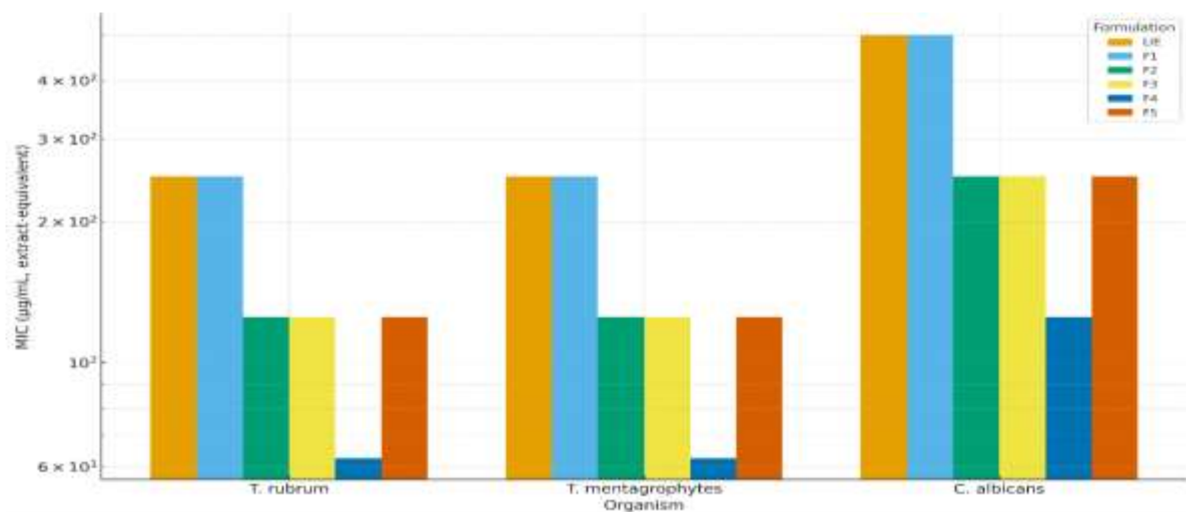

Figure 5. MICs of LIE and Lacquer Eluates against Fungi

3.5 Biofilm inhibition on nail fragments

3.5.1 Comparative viable counts

The untreated control group demonstrated dense colonization, yielding 5.6 ± 0.3 $\log_{10}$ CFU/disc after 48 h, confirming the ability of *T. rubrum* to form robust biofilms on keratinous substrates. Pre-treatment with the enhancer-free lacquer (F1) produced a modest reduction to 4.2 ± 0.2 $\log_{10}$ CFU/disc, corresponding to approximately a 1.4-log decrease compared to control. In contrast, the optimized formulation F4 achieved a significant reduction to 3.1 ± 0.2 $\log_{10}$ CFU/disc. This represented an additional 1.1-log reduction compared to F1 and nearly a 2.5-log reduction compared to untreated nails, with statistical significance ($p < 0.01$).

3.5.2 Implications for antifungal performance

Biofilms are a major therapeutic challenge in onychomycosis, as they confer enhanced tolerance to antifungals and protect embedded fungal cells. The superior performance of F4 highlights the benefit of combining HPCH with urea and thioglycolic acid, which likely facilitated deeper drug penetration into the nail plate and sustained lawsone delivery to the adherent fungal community. The keratolytic action of urea and disulfide disruption by TGA may also have contributed by altering the nail surface, reducing fungal anchorage sites, and exposing embedded biofilm cells to antifungal concentrations. The results indicate that while HPCH-based films alone (F1) provide some protection against fungal colonization, incorporation of enhancers (F4) substantially improves anti-biofilm efficacy. The nearly 2.5-log reduction relative to untreated control represents a biologically meaningful suppression of fungal load, consistent with improved antifungal susceptibility and permeation outcomes described earlier. This ability to inhibit biofilm formation is particularly valuable, as biofilm-associated persistence is a known contributor to therapeutic failure and recurrence in onychomycosis. Taken together, these findings reinforce the superiority of F4 as an optimized phytochemical-enriched lacquer, offering both fungicidal activity and significant anti-biofilm potential, thereby addressing two major obstacles in the topical management of nail fungal infections.

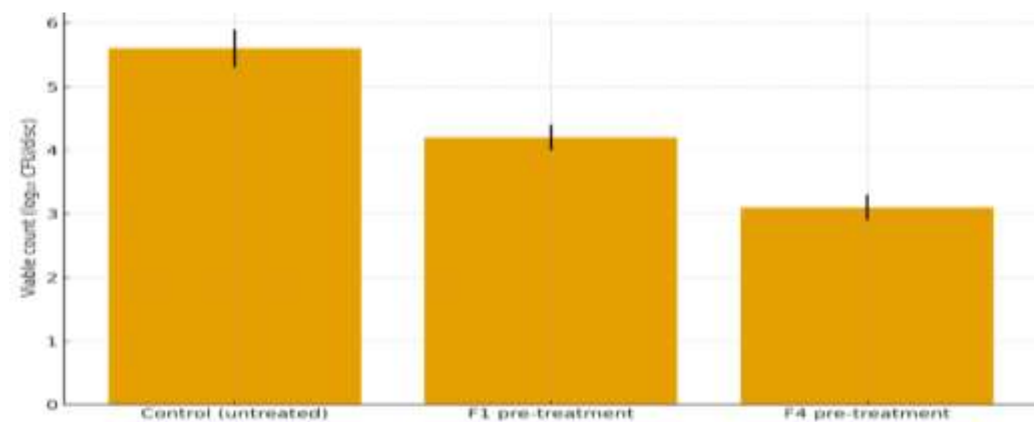

Figure 6. Biofilm Inhibition on Nail Fragments

3.6 Cytotoxicity on HaCaT keratinocytes

3.6.1 IC_{50} values and comparative tolerance

Resazurin assays on HaCaT keratinocytes demonstrated that bulk *L. inermis* extract (LIE) exhibited an IC_{50} of approximately 620 µg/mL. The optimized lacquer formulation (F4) showed an IC_{50} consistently greater than 500 µg/mL across independent replicates. These values are substantially higher than the antifungal MICs recorded against dermatophytes (62.5–125 µg/mL), indicating that effective antifungal concentrations are achieved well below cytotoxic thresholds. This suggests a favourable therapeutic index for topical application.

3.6.2 Mechanistic considerations

Lawsone, the primary naphthoquinone in *L. inermis*, is known to engage the aryl hydrocarbon receptor (AhR) signaling pathway in keratinocytes. While some naphthoquinones generate excessive reactive oxygen species leading to oxidative cytotoxicity, lawsone has been reported to exert milder redox effects. The lack of pronounced oxidative injury in keratinocytes at clinically relevant concentrations provides mechanistic support for its relative safety in topical nail formulations. Moreover, encapsulation within the HPCH film and controlled release from the lacquer likely further mitigate acute cytotoxic effects by preventing transient exposure to high local concentrations. The cytotoxicity findings reinforce the biocompatibility of F4, demonstrating that keratinocyte viability is preserved at and above concentrations required for antifungal efficacy. In practical terms, this safety margin is critical for long-term topical therapies, as repeated application is necessary in onychomycosis management. The results are consistent with prior reports of good skin tolerability of henna-based preparations and support the clinical translation of lawsone-containing lacquers. Collectively, these data confirm that F4 combines potent antifungal activity with acceptable safety for keratinocytes, satisfying two essential requirements for a viable therapeutic nail lacquer.

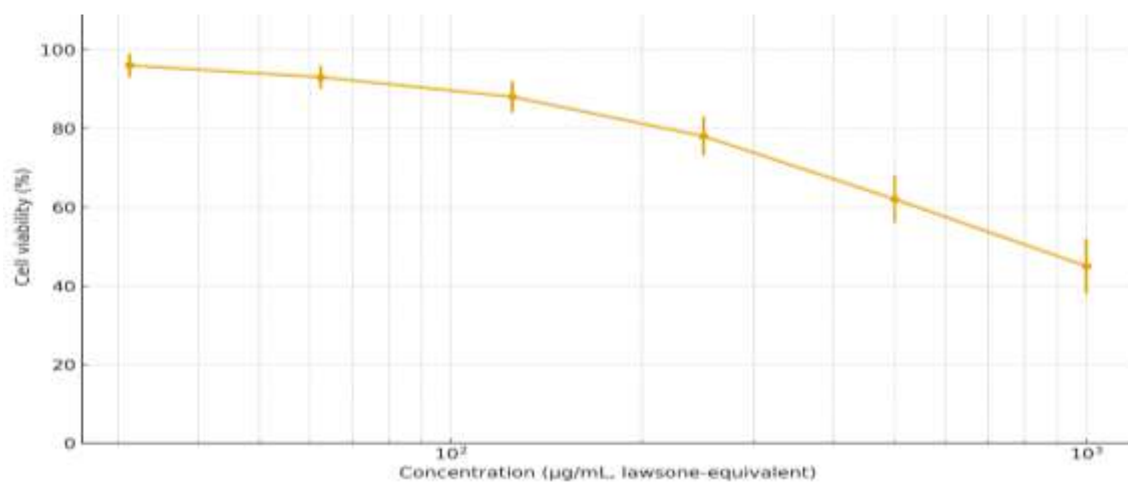

Figure 7. Cytotoxicity of LIE/F4 Eluate on HaCaT Keratinocytes

3.7 Stability

Accelerated stability testing of the optimized lacquer (F4) at 40 °C/75% RH for 12 weeks demonstrated excellent physicochemical resilience. The formulation retained its characteristic clear, amber appearance without signs of precipitation, phase separation, or color darkening. Viscosity showed only a minor increase of +4.8% compared to baseline, well within acceptable limits for semisolid and lacquer products. Such stability suggests that the HPCH polymer network and incorporated excipients maintained structural integrity under stress conditions, preventing premature degradation or cross-linking that could compromise film performance. Quantitative HPLC analysis confirmed that lawsone content remained at 98.2 ± 2.4% of the initial value after 12 weeks. This minimal loss highlights the inherent chemical stability of lawsone within the HPCH-based film, likely aided by the protective polymer matrix and use of amber containers that shielded from light-induced degradation. Retaining nearly full drug content over the test period ensures consistent therapeutic dosing during storage and use, addressing a key quality attribute for regulatory acceptance.

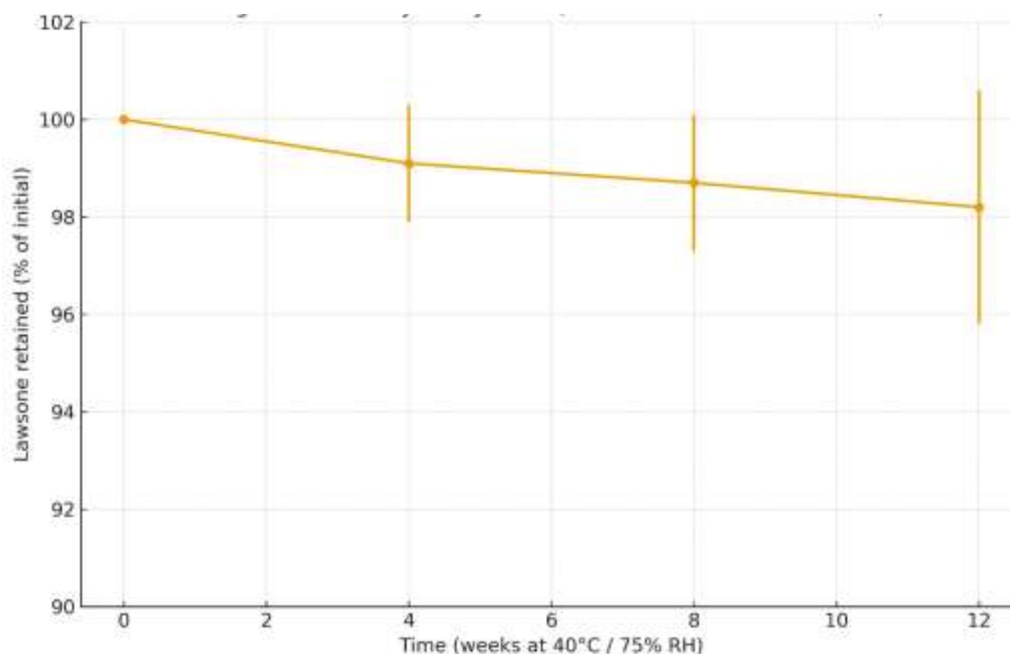

Figure 8. Stability Study of F4 (Lawsone Content Retention)

4. Discussion

This study demonstrates a rational, literature-guided approach to a phytochemical nail lacquer for onychomycosis. The selection of an HPCH vehicle capitalized on its water-soluble, keratin-affinitive, film-forming properties, which have supported the clinical performance of ciclopirox lacquers (Iorizzo et al., 2020). Our physicochemical data confirmed robust films with rapid drying, adequate solids, and strong adhesion—prerequisites for patient adherence and drug residence on the nail plate. Transungual permeation results aligned with the hypothesis that combining urea with TGA augments penetration. Both agents target the keratin barrier—urea through chaotropic, keratolytic action and TGA via reduction of disulfide bonds—with several studies reporting enhanced nail accumulation and flux of antifungals and model permeants when these enhancers are used, particularly in hydrated nails and diffusion-cell systems approximating clinical conditions (Khengar et al., 2007; Nair et al., 2009). Our Franz-cell design prioritized physiologic nail thickness and sink conditions as recommended by recent methodology reviews (Mertin & Lippold, 1997). F4's superior permeation translated to stronger antifungal effects (lower MICs and faster kill), consistent with drug availability within nail keratin. While standard CLSI M38-A2 microdilution remains the reference for antifungal susceptibility of molds, recognizing dermatophyte testing challenges (growth kinetics, inoculum dispersion) is crucial; our adherence to CLSI principles enhanced comparability and interpretability (CLSI, 2008a; CLSI, 2008b).

The choice of *L. inermis* was supported by evidence that henna/lawsone exhibits antifungal activity against clinical dermatophyte isolates, and that naphthoquinone scaffolds possess broad antimicrobial properties with redox-active mechanisms (Chaudhary et al., 2010; Kumar et al., 2011). We standardized lawsone content to reduce phytochemical variability. Although lawsone can engage AhR signaling in keratinocytes, literature suggests comparatively modest oxidative cytotoxicity vs other naphthoquinones at similar ranges; our HaCaT data are congruent with a reasonable topical safety margin (Bickers & Calow, 2003). Nevertheless, clinical caution is warranted in individuals with G6PD deficiency due to rare hemolytic events associated with henna exposure (Patel et al., 2017), and adulterants such as p-phenylenediamine must be excluded by quality controls (Brancaccio et al., 1997). Compared with marketed lacquers (amorpholine or hydroxypyridone mechanisms), a lawsone-standardized HPCH lacquer offers a plant-derived alternative that may appeal to patients avoiding long-term systemic therapy or those seeking adjunctive options. Literature on medicated lacquers shows strong real-world acceptability and efficacy, particularly when film technology optimizes deposition and when keratolytic pretreatments are used (Rigopoulos et al., 2008; Gupta et al., 2020). Our formulation embeds keratolytics to reduce reliance on separate pretreatment steps.

Limitations:

The work is in vitro. Although human nail clippings are a relevant barrier, infected nails may have altered porosity and onycholysis; incorporating ex-vivo infected nails and microscopy (e.g., CLSM mapping of fluorescent probes) would strengthen translational inference (Ghannoum et al., 2013). Repeated-dose cycling and wash-off studies are also needed to model real-life application. Finally, establishing clinical MIC–PK/PD relationships for botanical actives remains challenging and will require carefully designed trials (Turner et al., 2016).

Future directions:

Pairing the lacquer with physical enhancement (micro-abrasion or fractional laser) may further boost deposition at lower enhancer levels, as reported for amorolfine with TGA/laser combinations (Lim et al., 2014). Exploring synergism with low-dose synthetic antifungals, assessing dermatophyte biofilm disruption in dynamic flow models, and conducting pilot clinical studies against mild-to-moderate distal lateral subungual onychomycosis would be logical next steps.

5. Conclusion

A phytochemical-enriched HPCH nail lacquer standardized to lawsone from *L. inermis* and fortified with urea and thioglycolic acid demonstrated favorable film properties, enhanced transungual permeation, and meaningful antifungal activity against *T. rubrum*, *T. mentagrophytes*, and *C. albicans* in vitro. The optimized formula (F4) achieved a three-fold increase in 24 h nail permeation compared with enhancer-free lacquer and produced fungicidal time-kill kinetics. These findings, consistent with contemporary understanding of ungual delivery and enhancer mechanisms, justify ex-vivo infected nail studies and early clinical evaluation as a botanical, patient-friendly option for onychomycosis management.

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