Infection and Drug Resistance

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ORIGINAL RESEARCH

Formulation and Evaluation of a Licorice-Resveratrol Lollipop for Targeting *Streptococcus mutans* Biofilm and Antimicrobial Resistance

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Background: *Streptococcus mutans* is a key pathogen in dental caries, and the development of novel antimicrobial formulations is crucial to combat its resistance. This study aimed to evaluate a licorice-resveratrol medicated lollipop formulation (LRML) for its antimicrobial and anti-biofilm activity against *S. mutans*.

Methods: The LRML was developed using a heating and congealing method, incorporating licorice extract (5% w/w) and resveratrol (2% w/w) in a sucrose-based matrix. The physicochemical properties of the formulation, including hardness, drug content uniformity, moisture content, and dissolution profile, were evaluated. The antimicrobial activity was assessed through Minimum Inhibitory Concentration (MIC), Minimum Bactericidal Concentration (MBC), and time-kill assays. Anti-biofilm activity was evaluated using a crystal violet assay. The stability of the formulation was determined under accelerated conditions.

Results: The LRML formulation showed efficient drug release, with formulation number LRML-7 demonstrating 96.87% release within 45 minutes. The antimicrobial tests revealed significant bactericidal effects against *S. mutans* at concentrations above 0.2 µg/mL, with a notable reduction in bacterial growth in time-kill assays. The formulation also demonstrated substantial inhibition of biofilm formation at both MIC and Minimum Bactericidal Concentration (MBC) levels. Stability studies confirmed that the formulation retained its physicochemical properties over three months.

Conclusion: The LRML exhibited promising antimicrobial and anti-biofilm activities against *S. mutans*, suggesting its potential as a novel therapeutic option for managing dental infections. Further clinical studies are required to optimize the formulation's efficacy and clinical applicability.

Keywords: licorice, resveratrol, antimicrobial, anti-biofilm, *Streptococcus mutans*, drug release, stability

Introduction

Dental caries remains a global public health challenge, affecting 2.5 billion people worldwide, with untreated cavities prevalent in nearly 30% of adults and 530 million (approximately 60% to 90% of school-aged) children as per recent WHO reports.[1] The disease is characterized by the demineralization of tooth enamel and dentin due to acidic byproducts generated by cariogenic bacteria, primarily *Streptococcus mutans* and *Streptococcus sobrinus*.[2] These bacteria metabolize fermentable carbohydrates (eg, sucrose, glucose, and fructose) into organic acids, creating a low-pH environment that promotes enamel erosion and cavity formation.[3] Despite advances in preventive measures such as fluoride therapy and dental sealants, recurrent caries remains a significant challenge, particularly in high-risk populations.[4] Current restorative treatments, including fillings and root canals, often fail to address the underlying microbial etiology, necessitating

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innovative therapeutic strategies that directly target cariogenic pathogens while minimizing antimicrobial resistance.[5] Recent developments in drug delivery systems (DDS) have introduced novel approaches for localized oral disease management.[6,7] Among these, suckable formulations such as medicated lollipops and lozenges—offer distinct advantages by enabling sustained release of bioactive compounds directly at the site of infection.[8] These formulations enhance drug retention in the oral cavity, improving therapeutic efficacy against bacterial biofilms while ensuring patient compliance, particularly in pediatric and geriatric populations.[9] Previous studies have explored various natural compound-loaded DDS, including mouthwashes and gels; however, few have combined natural antimicrobials within a sustained-release lollipop matrix, highlighting an opportunity for innovation[9,10] Natural phytochemicals with antimicrobial and anti-biofilm properties have gained attention as promising alternatives to conventional antibiotics, which face increasing resistance concerns. Licorice (Glycyrrhiza glabra), a well-documented herb in traditional medicine, contains bioactive compounds such as glycyrrhizin, glabridin, and flavonoids, which exhibit broad-spectrum antimicrobial and anti-inflammatory effects.[11] Studies indicate that licorice extract specifically inhibits *S. mutans* growth and biofilm formation, making it a viable candidate for caries prevention.[12] Similarly, resveratrol (3,5,4′-trihydroxystilbene), a polyphenol found in grapes and berries, demonstrates potent antioxidant, anti-inflammatory, and antimicrobial properties.[13] Research suggests that resveratrol disrupts *S. mutans* virulence by interfering with acid production and glucan synthesis, key factors in cariogenic biofilm development.[14] This study focuses on formulating and evaluating a licorice-resveratrol loaded lollipop designed to target *S. mutans* biofilms and mitigate antimicrobial resistance. By combining these natural compounds, the formulation aims to synergistically enhance antibacterial efficacy while leveraging the prolonged mucosal adhesion of a suckable delivery system.[15] The lollipop's-controlled release mechanism is expected to maintain effective drug concentrations in saliva, ensuring sustained action against pathogenic bacteria. Additionally, the anti-inflammatory and antioxidant properties of both licorice and resveratrol may promote oral tissue healing, offering a multifunctional approach to caries management. Compared to previous formulations such as mouthwashes or gels loaded with single phytochemicals, the present LRML offers a novel and patient-friendly alternative that ensures localized, prolonged delivery of dual active agents with improved bioavailability and compliance. The rising global burden of dental caries, coupled with the limitations of existing treatments, underscores the need for innovative, non-invasive therapies.[16] We hypothesize that the LRML formulation will synergistically inhibit S. mutans biofilms, reduce bacterial growth, and provide an effective multifunctional therapeutic strategy for caries prevention. If successful, this LRML could serve as a scalable, patient-friendly intervention, particularly for high-risk groups. Future research will explore its clinical efficacy, safety, and potential for reducing antibiotic overuse in dentistry.

Materials and Methods

This study was conducted in compliance with Good Laboratory Practice (GLP) guidelines of the Sant Gajanan Maharaj College of Pharmacy. No human or animal subjects were involved in the experimental procedures. All experiments, including physicochemical evaluation, drug content analysis, dissolution studies, and antimicrobial/anti-biofilm assays, were performed in triplicate (n = 3) unless otherwise stated, to ensure reliability and reproducibility of the data. Licorice extract used was a standardized Glycyrrhiza glabra root extract (Batch No. LH-2305) was procured from Aromatic Herbals (Pune, India). Resveratrol (≥99% purity, Cat. No. R5010) and citric acid anhydrous were obtained from Sigma-Aldrich (St. Louis, MO, USA). Methyl cellulose (MC, viscosity 4000 cP, Batch No. MC-0412) and hydroxypropyl methylcellulose (HPMC K15M, Batch No. HPMC-0923) were sourced from Research-Labs Fine Chem Industries (Mumbai, India) and Molychem (Mumbai, India), respectively. Food-grade sucrose and corn syrup were purchased from a local supplier and verified for purity. All chemicals and reagents were of analytical grade, and ultrapure water (Milli-Q, 18.2 MΩ·cm) was used for all preparations.

Liquorice-Resveratrol Medicated Formulation

The medicated Liquorice-Resveratrol were formulated using a heating and congealing method.[17] Sucrose (50% w/w) was dissolved in ultrapure water under constant stirring (500 rpm) at 85 ± 2°C using an IKA RCT Basic digital hotplate. Corn syrup (20% w/w) was added when the temperature reached 110 ± 1°C, monitored with a calibrated thermocouple. The mixture was heated to 156 ± 0.5°C to form a viscous syrup, confirmed using a candy thermometer. The hot syrup

(154.4°C) was transferred to a vacuum chamber and maintained at 274 mmHg for 30 ± 2 min to reduce moisture content to ≤1.5%, as determined by Karl Fischer titration (Metrohm 870, Metrohm AG, Herisau, Switzerland). The dehydrated syrup was cooled to 90°C on a water-jacketed stainless-steel table (214 ft²) and mixed with licorice extract (5% w/w), resveratrol (2% w/w), citric acid (1% w/w), and polymers (3% w/w). The mixture was poured into calibrated molds, air-cooled, and dried in a desiccator for 24 h. The final products were individually wrapped in aluminum foil to maintain stability.

Pre-Formulation Characterization

Comprehensive pre-formulation studies were conducted to evaluate the physicochemical compatibility of the drug and excipients prior to formulation development.[18] Fourier-transform infrared spectroscopy (FTIR) was employed to detect potential interactions between the active pharmaceutical ingredient (API) and excipients by analyzing characteristic functional group vibrations. FTIR spectra were recorded using a Shimadzu IRSpirit spectrometer (Shimadzu Corporation, Kyoto, Japan). The samples were uniformly mixed with potassium bromide (KBr) in a 1:100 ratio and compressed into transparent discs using a hydraulic press. Spectral acquisition was performed over the wavenumber range of 4000 to 400 cm^{-1} at a resolution of 4 cm^{-1}. Thermal behavior and compatibility were further investigated using Differential Scanning Calorimetry (DSC) on a Shimadzu DSC-60 instrument (Shimadzu Corporation). Approximately 5–10 mg of each sample was accurately weighed and hermetically sealed in standard aluminum pans. The analysis was carried out over a temperature range of 30°C to 300°C at a linear heating rate of 10°C/min under a nitrogen atmosphere, with a constant purge rate of 50 mL/min. Thermograms were analyzed for shifts, disappearance, or emergence of peaks indicative of potential interactions or changes in crystallinity. Additionally, the UV-visible absorption spectrum of the licorice extract was recorded using a Shimadzu UV-2550 spectrophotometer (Shimadzu Corporation). The sample was dissolved in distilled water, and the absorbance was measured across the wavelength range of 200 to 400 nm. This analysis facilitated the identification of characteristic chromophores and helped establish the preliminary spectral profile of the extract, useful for subsequent analytical method development.

Evaluation of Formulations

The hardness of the lollipops was measured using a Monsanto hardness tester (MH-200, LabIndia, Mumbai, India), with results expressed in kg/cm² (n = 5). Weight variation was determined by individually weighing 10 randomly selected lollipops using an analytical balance (Sartorius CPA225D, Sartorius AG, Göttingen, Germany; readability 0.01 mg). Moisture content was assessed by drying 1 g of crushed lollipop in a desiccator for 24 h and calculating the weight loss. Drug content uniformity was analyzed by dissolving crushed lollipops in phosphate buffer (pH 6.7) and measuring absorbance at 254 nm (Shimadzu UV-2550). In *vitro-drug* release studies were performed using a USP-II dissolution apparatus (Electrolab TDT-08L, Electrolab India Pvt. Ltd., Mumbai, India) with 100 mL of phosphate buffer (pH 6.7) at 37 ± 0.5°C and 100 rpm. Samples (5 mL) were withdrawn at 5-min intervals and replaced with fresh medium (indicated LRML 1 to 9). Drug concentration was determined spectrophotometrically at 254 nm.

Stability Studies

The optimized lollipop formulations were stored in aluminum collapsible tubes under accelerated conditions (40 ± 2°C, 75 ± 5% RH) for 3 months. Samples were evaluated monthly for hardness, drug content, and dissolution properties to assess stability.[19]

Antimicrobial and Antibiofilm Activity

All assays were validated using *S. pneumoniae* ATCC 49619 as a control strain for MIC determination as per CLSI M100 standards.

Minimum Inhibitory Concentration (MIC) Determination

The antimicrobial potency of the formulation against *S. mutans* (ATCC 35668) was evaluated by determining the MICs using the standardized microbroth dilution technique in accordance with the Clinical and Laboratory Standards Institute

(CLSI) M100 guidelines 34th addition.[20] Bacterial suspensions were adjusted to a 0.5 McFarland standard (~1×10^8 CFU/mL) using sterile saline and further diluted to yield a final inoculum of 5×10^5 CFU/mL in each well. Serial two-fold dilutions of the test formulation were prepared in Thioglycollate broth to achieve final concentrations ranging from 100 to 0.2 µg/mL in 96-well microtiter plates. Thioglycollate broth was selected for MIC determination because it creates a reducing, oxygen-depleted environment that supports the optimal anaerobic growth of S. mutans. Although Brain Heart Infusion (BHI) broth is widely used for S. mutans cultivation, Thioglycollate broth is also commonly employed in antimicrobial susceptibility testing of anaerobic bacteria due to its effective oxygen-scavenging properties. We have added this explanation in the Methods section to clarify the rationale behind our media selection. Plates were incubated at 37°C for 48 hours under strict anaerobic conditions using an anaerobic jar with gas packs. The MIC was defined as the lowest concentration of the formulation that completely inhibited visible bacterial growth when observed under standard lighting conditions.

Time-Kill Assay

The bactericidal kinetics of the formulation were further investigated through a time-kill assay. *S. mutans* cultures were exposed to the formulation at concentrations corresponding to 1×, 2×, and 4× the MIC value. The treated and untreated control cultures were incubated at 37°C under anaerobic conditions. At predetermined time intervals (0, 2, 4, 8, 12, and 24 hours), 100 µL aliquots were withdrawn, serially diluted in sterile phosphate-buffered saline (PBS), and plated on Mueller-Hinton agar. The agar plates were incubated for 48 hours anaerobically, and the resulting colonies were counted manually. The bactericidal activity was expressed as the $\log_{10}$ reduction in colony-forming units per milliliter ($\log_{10}$ CFU/mL). A ≥ 3 $\log_{10}$ reduction from the initial CFU/mL was considered indicative of bactericidal activity.

Anti-Biofilm Activity

The anti-biofilm efficacy of the formulation was assessed using the crystal violet staining assay.[21] *S. mutans* biofilms were established by inoculating brain heart infusion (BHI) broth supplemented with 1% sucrose in sterile 96-well polystyrene microplates, followed by incubation at 37°C for 72 hours under anaerobic conditions to allow mature biofilm formation. Post-incubation, planktonic cells were gently removed, and wells were washed thrice with sterile PBS. The preformed biofilms were then treated with MIC and MBC concentrations of the formulation for 24 hours. After treatment, the wells were washed to remove non-adherent cells and stained with 0.1% (w/v) crystal violet for 15 minutes. Excess stain was removed by rinsing with distilled water, and the bound dye was solubilized in 95% ethanol. Absorbance was measured at 570 nm using a BioTek Synergy H1 microplate reader (BioTek Instruments, Inc., Winooski, VT, USA). A reduction in absorbance compared to untreated controls indicated inhibition of biofilm biomass.

Results

Pre-Formulation Characterization

UV-Visible Spectroscopy

The UV-Visible spectrum of the licorice extract in phosphate buffer (pH 6.8) revealed a well-defined absorption maximum (λmax) at 277 nm (Figure 1), indicating the presence of conjugated π-electron systems, most likely from flavonoids and glycyrrhizic acid derivatives known to be present in licorice. The linear regression of absorbance versus concentration yielded a strong correlation ($y = 0.0009x$, $R^2 = 0.9809$), confirming the extract's suitability for quantitative analysis. The absence of secondary peaks or broadening (200–400 nm) further validated the sample's chemical homogeneity and lack of UV-active impurities.

FTIR Spectroscopy

The FTIR analysis provided insight into the molecular fingerprint of the individual components and their physical mixture. The spectrum of pure licorice extract (Figure 2A) exhibited characteristic absorption bands at 3286 cm^{-1} (O–H stretching, indicative of hydroxyl groups), 2927 cm^{-1} (aromatic C–H stretching), and 1639 cm^{-1} (C=O stretching vibration), aligning with known functional groups in glycyrrhizic acid and other polyphenolic constituents. These findings confirm the integrity of the bioactive compounds within the extract. Erythritol (Figure 2B) demonstrated

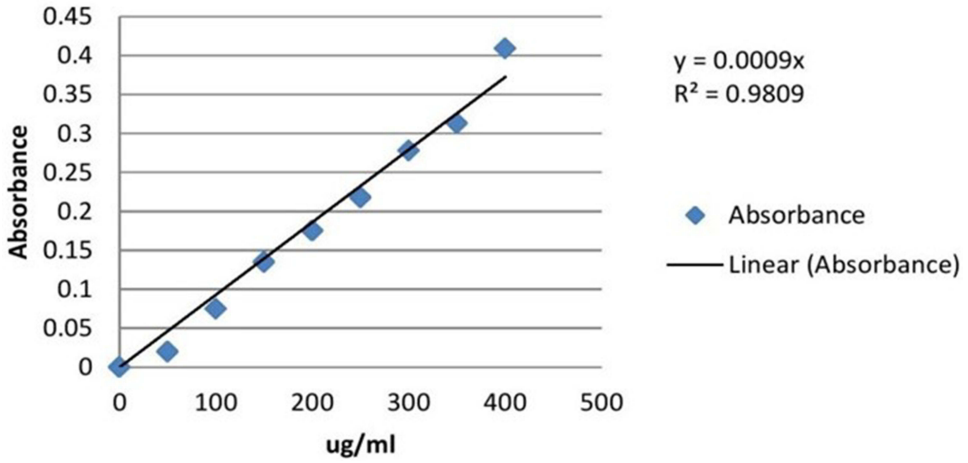

Figure 1 UV-Visible Spectrum of Licorice Extract. The UV-Visible spectrum of the licorice extract in phosphate buffer (pH 6.8) exhibited a well-defined absorption maximum (λmax) at 277 nm, indicating the presence of conjugated π-electron systems, characteristic of flavonoids and glycyrrhizic acid derivatives.

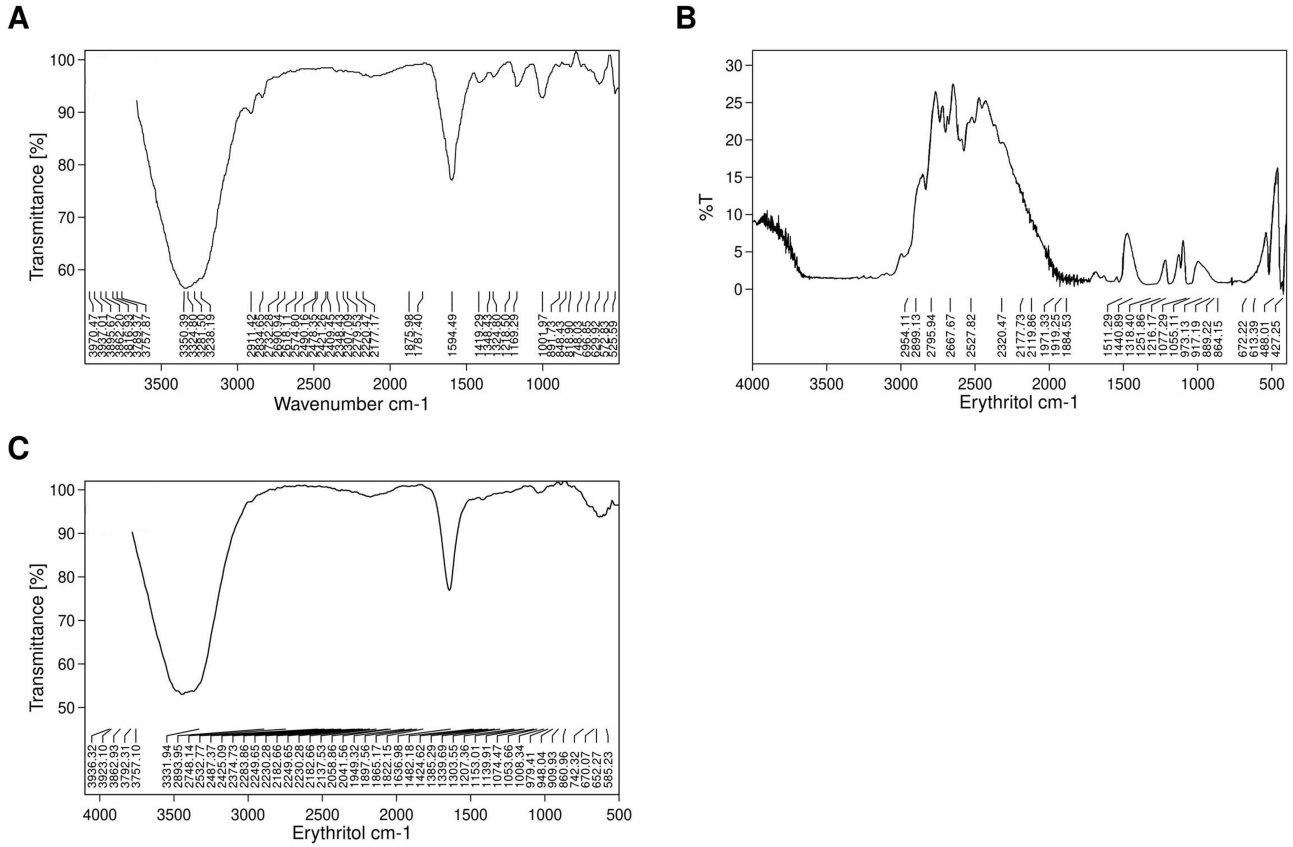

Figure 2 FTIR Spectra of Licorice Extract, Erythritol, and the Physical Mixture of LRML. (**A)** The FTIR spectrum of pure licorice extract; (**B)** The FTIR spectrum of erythritol and (**C)** The FTIR spectrum of the physical mixture (LRML).

sharp and intense peaks at 2954 cm^{-1} (O–H stretching of alcohol groups) and a significant peak at 1251 cm^{-1} corresponding to C–O stretching vibrations. The clarity and consistency of these peaks confirmed the chemical purity and crystalline nature of erythritol. Importantly, the FTIR spectrum of the physical mixture LRML (Figure 2C) retained all the significant absorption bands from both the drug and excipient without any noticeable shift, disappearance, or formation of new peaks. The O–H and C=O bands remained prominent at 3286 cm^{-1} and 1636 cm^{-1}, respectively. These

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observations suggest the absence of any significant physicochemical interaction between the licorice extract and erythritol, indicating good compatibility.

Differential Scanning Calorimetry (DSC)

DSC analysis was conducted to assess the thermal behavior and compatibility of the drug and excipient. The thermogram of pure licorice extract (Figure 3A) showed a sharp endothermic peak at 95.77°C, which corresponds to its melting point and indicates the presence of a crystalline structure. The narrow, symmetrical peak shape also reflects a well-defined and pure thermal event with minimal impurity interference. Erythritol (Figure 3B) exhibited a distinct exothermic peak at 120.82°C, a transition associated with its crystalline-to-amorphous conversion, confirming its stable polymorphic form under the experimental conditions. For the physical mixture (LRML), the DSC thermogram (Figure 3C) displayed a single endothermic peak at 97.00°C, which is closely aligned with the melting point of licorice extract. The absence of new or shifted peaks in the thermal profile suggests that no interaction or degradation occurred during mixing. Furthermore, the lack of peak broadening or multiple transitions indicates the physical mixture remained thermodynamically stable and retained the crystalline nature of its constituents without forming new polymorphic species.

Formulation Evaluation

Physicochemical Properties

The tablet formulations were subjected to a comprehensive assessment of key physicochemical parameters. Hardness, an indicator of mechanical strength, was measured using the Monsanto hardness tester (MH-200). Among the tested batches, formulation F9 demonstrated the highest hardness value of 12.07 ± 0.22 kg/cm², which was attributed to its higher polymer concentration, particularly 2% w/w hydroxypropyl methylcellulose (HPMC). This increase in HPMC

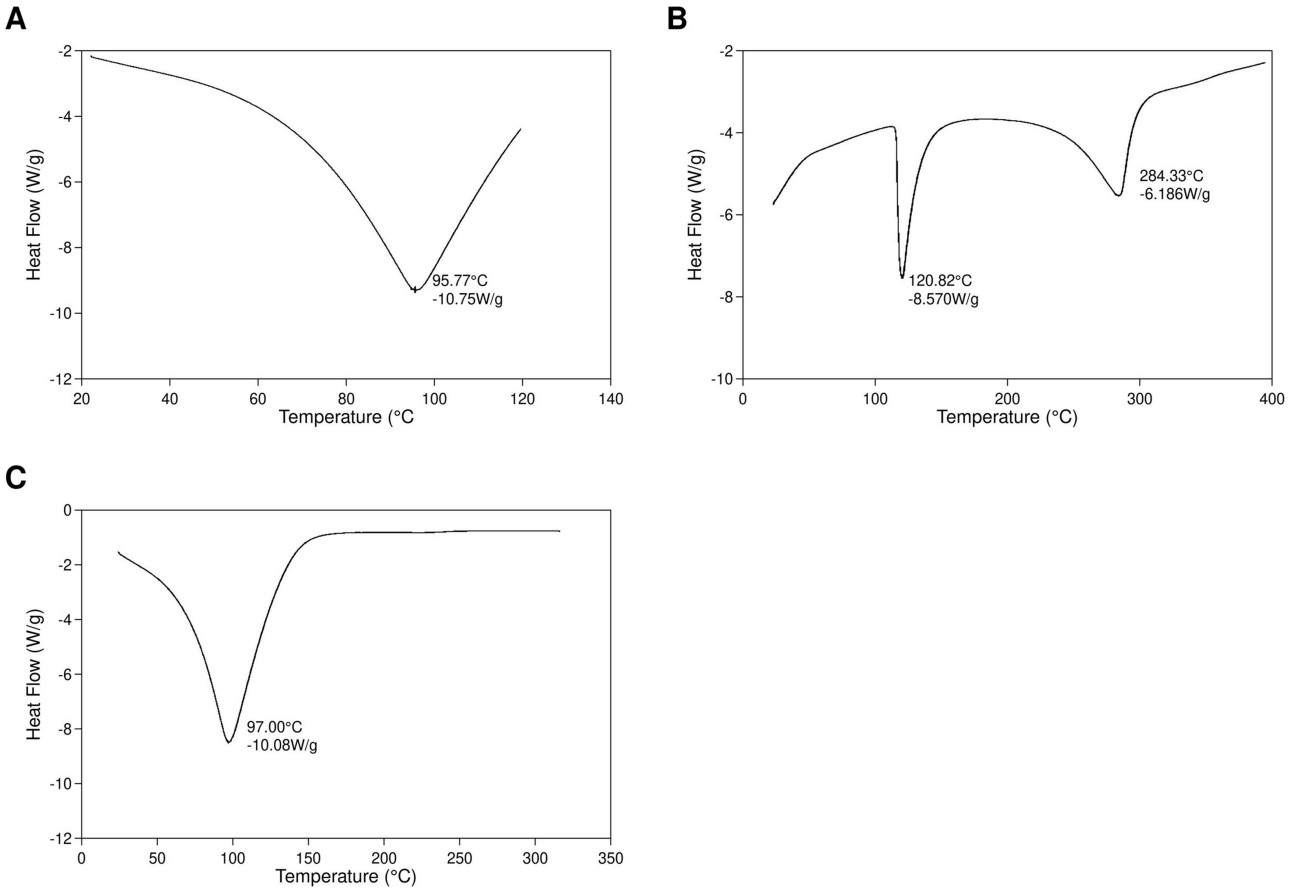

Figure 3 DSC Thermograms of Licorice Extract, Erythritol, and the Physical Mixture of LRML. (**A**) The DSC thermogram of pure licorice extract; (**B**) The DSC thermogram of erythritol; (**C**) The DSC thermogram of the physical mixture (LRML).

Table 1 Physicochemical Evaluation of LRML

Batch	Hardness (kg/cm²)	Drug Content (% w/w)	Weight Variation (g)	Friability (% loss)	Moisture Content (% w/w)
LRML1	9.53±0.42	51.22±0.02	5.04±0.05	0.92 ±0.01	0.49 ±0.01
LRML2	9.77±0.11	62.33±0.14	5.05±0.21	0.93 ±0.01	0.49 ±0.01
LRML 3	10.00±0.15	96.00±0.12	5.05±0.04	0.94 ±0.0	0.45 ± 0.01
LRML 4	9.64±1.11	90.22±0.54	4.98±0.65	0.93 ±0.01	0.47 ± 0.03
LRML 5	10.02±0.42	85.00 ±0.65	5.33±0.26	0.93 ±0.01	0.48± 0.00
LRML 6	10.29±0.15	62.11±0.24	5.12±0.2	0.94 ±0.01	0.49 ±0.01
LRML 7	10.56±0.39	96.00±0.87	5.03±0.04	0.92 ±0.02	0.49 ±0.03
LRML 8	11.49±0.10	81.00±0.45	4.79±0.05	0.93 ±0.02	0.47 ± 0.01
LRML 9	12.07±0.22	83.11±0.23	4.90±0.15	0.92 ±0.01	0.44 ±0.02

Abbreviation: LRML, Liquorice-Resveratrol Medicated Lollipop.

content likely enhanced the binding properties of the matrix, contributing to improved compressibility and cohesion (Table-1). Drug content uniformity was evaluated using UV spectrophotometry at 254 nm after dissolution in phosphate buffer (pH 6.8). The results revealed a wide but consistent range of drug content between 51.22% and 96.00%, with standard deviations ranging from ±0.02 to ±0.87, indicating acceptable uniformity across all batches and compliance with pharmacopeial limits. The residual moisture content, assessed via Karl Fischer titration using the Metrohm 870 titrator, confirmed that all formulations retained moisture levels below 1.5% following vacuum drying at 274 mmHg for 30 minutes. These values indicate adequate desiccation and support the stability of the tablets by reducing the risk of hydrolytic degradation.

In-vitro Drug Release

Dissolution profiles were evaluated using the USP type II (paddle) dissolution apparatus (Electrolab TDT-08L) operated at 100 rpm in phosphate buffer (pH 6.7) maintained at 37°C. Among the different formulations, LRML-7 (containing 1.2% HPMC and 1.5% methylcellulose) exhibited the most efficient release pattern, reaching 96.87% cumulative drug release within 45 minutes (Figure 4). This suggests that the specific polymer blend in LRML7 effectively modulated the hydration and erosion dynamics of the tablet matrix, facilitating rapid and complete release. Mathematical modeling of the drug release data using polynomial regression and analysis of variance (ANOVA, $p < 0.05$) confirmed that the release kinetics were significantly influenced by the composition variables. The developed model exhibited a high predictive accuracy with a coefficient of determination (R^2) of 0.98, indicating a strong correlation between experimental and predicted release values (Figure 5A and B).

Stability Studies

Stability testing under accelerated conditions (40°C ± 2°C and 75% ± 5% relative humidity for three months) was conducted in accordance with ICH Q1A (R2) guidelines. The physical integrity of the tablets was largely maintained throughout the study. For instance, the hardness of the selected formulation declined only marginally from 10.56 ± 0.39 kg/cm² on Day 0 to 9.87 ± 0.09 kg/cm² at the end of three months, indicating robust mechanical stability under stress conditions (Table 2). The formulation retained its therapeutic performance over time, with a drug release efficiency of 95.02 ± 0.24% at Month 3, well within acceptable pharmacopeial thresholds. These results validate the formulation's resilience and its potential for long-term storage without compromising quality or efficacy.

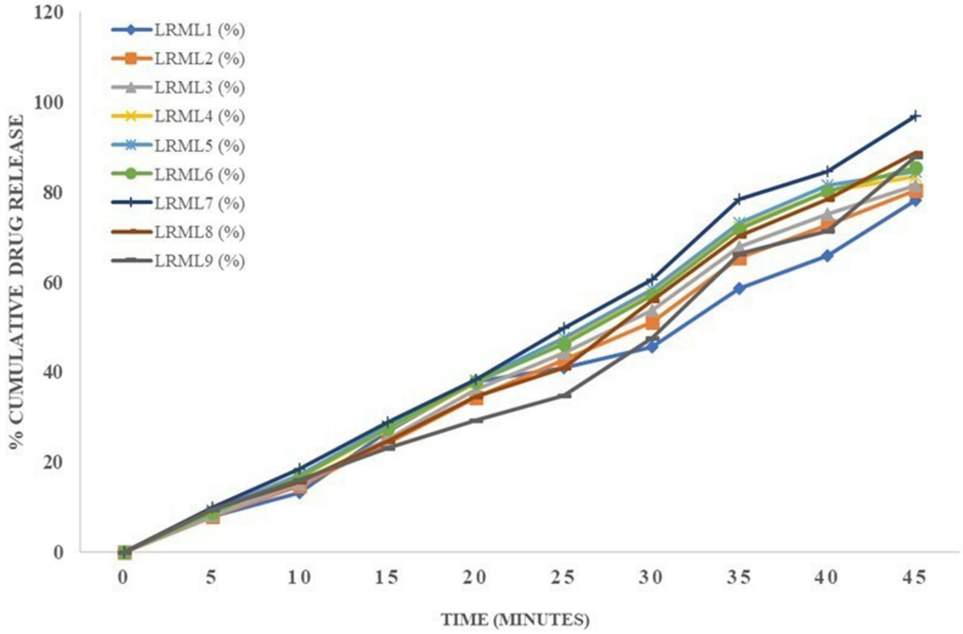

Figure 4 In-Vitro Drug Release Profile of LRML.

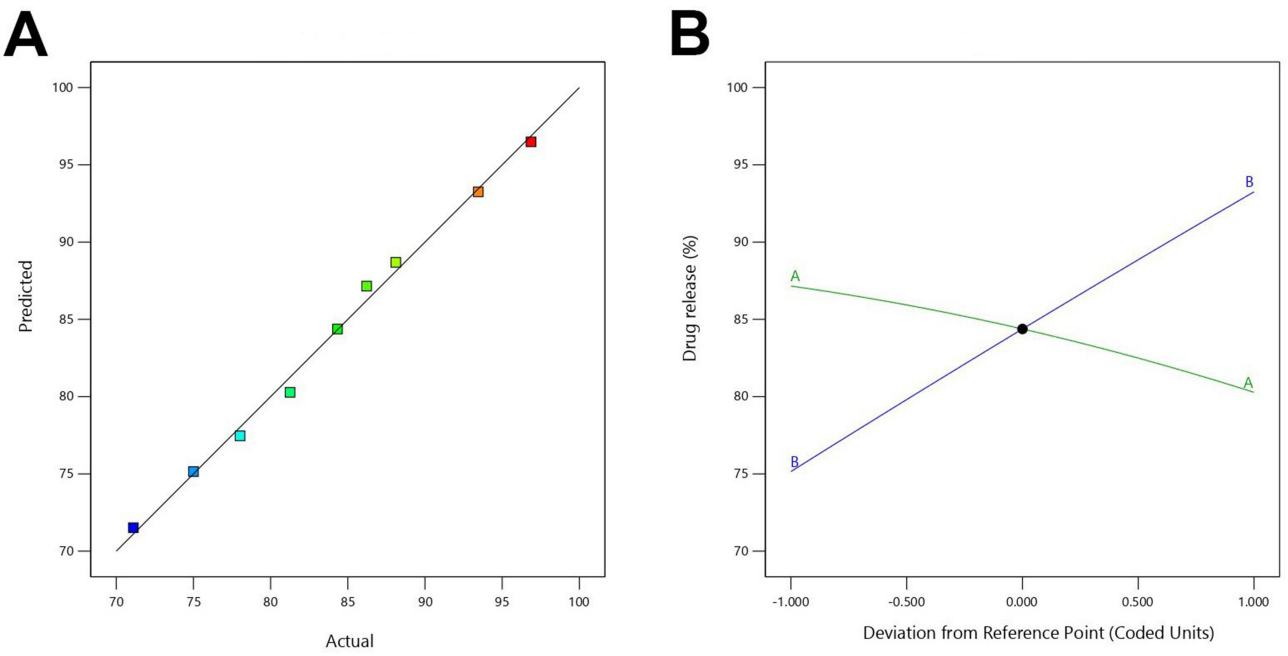

Figure 5 Drug Release Kinetics Model. (**A**) Polynomial regression analysis of cumulative drug release from LRML-7, illustrating the model's fit to the experimental dissolution data. (**B**) Correlation plot comparing experimental versus predicted drug release values, demonstrating a strong predictive relationship (R^2 = 0.98).
Note: The model confirms that formulation variables significantly influenced release kinetics (ANOVA, $p < 0.05$).

Antimicrobial Activity

Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

The antimicrobial potency of the liquorice extract and the liquorice-based medicated lollipop (LRML) was evaluated against *S. mutans* (ATCC 35668) using the standardized microbroth dilution method in accordance with Clinical and Laboratory Standards Institute (CLSI) M100 guidelines. Following 48 hours of anaerobic incubation at 37°C, the

Table 2 Stability Profile of Optimized LRML Formulation Under Accelerated Conditions (40°C ± 2°C/75% ± 5% RH)

Stability Condition	Sampling Interval (months)	Hardness (Kg/cm^3)	Drug Release (%)
40°C ± 2°C and 75% ± 5% RH	0	10.56±0.39	96.87± 0.94
	1	10.43±0.31	96.51± 0.85
	2	10.12±0.22	95.49 ± 0. 43
	3	9.87±0.09	95.02 ±0.24

liquorice extract demonstrated an MIC of 12.5 µg/mL and an MBC of 25 µg/mL, indicating a moderate inhibitory and bactericidal effect (Table 3). Conversely, the LRML formulation exhibited significantly enhanced antimicrobial activity, with both MIC and MBC values determined to be 0.2 µg/mL. This 62.5-fold improvement in MIC compared to the crude extract suggests that formulation into a solid dosage form augmented the bioavailability and/or retention of the active phytoconstituents at the site of action. The uniform and low MIC and MBC values further support a potent and consistent antibacterial effect of the formulated product.

Time-Kill Kinetics

To elucidate the bactericidal dynamics over time, a time-kill assay was performed using the LRML formulation at concentrations equivalent to 1×, 2×, and 4× the MIC. Bacterial suspensions of *S. mutans* (ATCC 35668) were sampled at predetermined intervals (0, 2, 4, 8, 12, and 24 h), plated on Mueller-Hinton agar, and viable colonies were enumerated. Exposure to 4× MIC of LRML led to a ≥3 $\log_{10}$ CFU/mL reduction in bacterial count within 8 hours (Figure 6), signifying a bactericidal effect. The rapid decline in bacterial load underscores the formulation's capacity for efficient microbial clearance, which is critical for applications in oral health where brief contact times with therapeutic agents are typical.

Table 3 MIC and MBC of Liquorice Extract and LRML Against Streptococcus mutans

Concentration (µg/mL)	Growth with Liquorice Extract	Bactericidal Effect (Extract)	Growth with LRML	Bactericidal Effect (LRML)
100	No	Yes	No	Yes
50	No	Yes	No	Yes
25	No	**Yes (MBC)**	No	**Yes (MBC)**
12.5	**No (MIC)**	No	No	Yes
6.25	Yes	No	No	Yes
3.125	Yes	No	No	Yes
1.56	Yes	No	No	Yes
0.78	Yes	No	No	Yes
0.39	Yes	No	No	Yes
0.2	Yes	No	**No (MIC)**	Yes

Notes: *"No" in Growth = no visible bacterial growth (ie, inhibition); "Yes" in Bactericidal Effect = no colony growth on subculture (ie, bactericidal); MIC and MBC values are bolded for emphasis.

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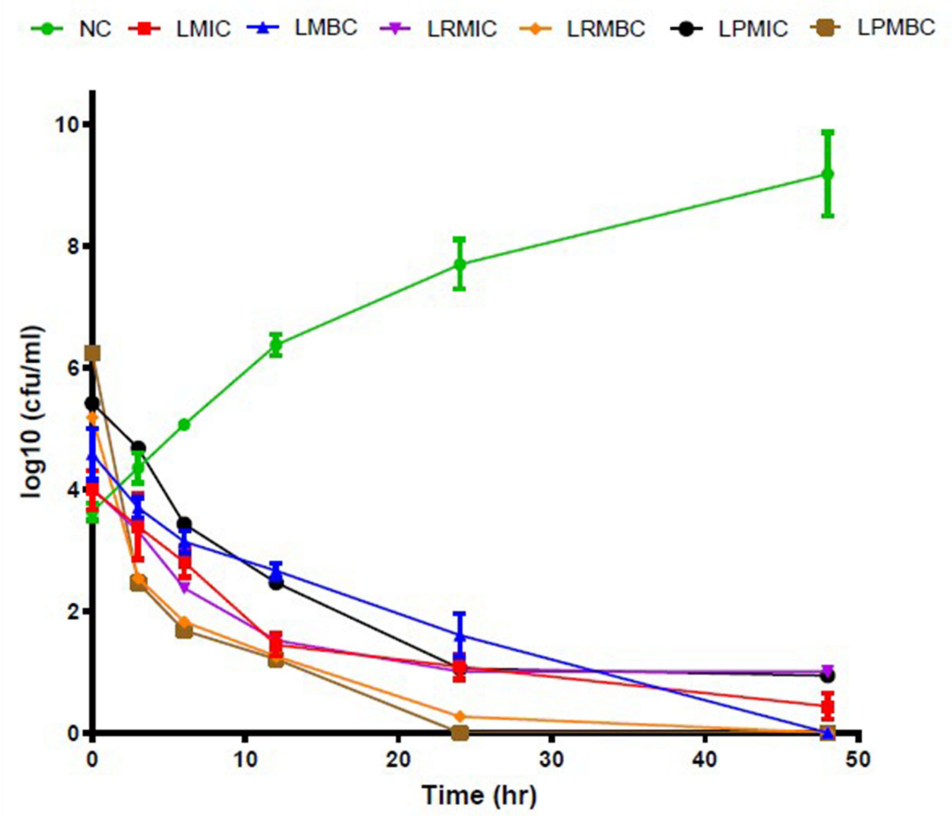

Figure 6 Time-Kill Kinetics of LRML Against *S. mutans*.

Inhibition of Biofilm Formation

The anti-biofilm efficacy of LRML was assessed using the crystal violet staining method to quantify biomass of *S. mutans* biofilms formed over 72 hours in brain heart infusion (BHI) broth. Biofilms treated with the LRML formulation at MIC and MBC concentrations for 24 hours exhibited a marked reduction in total biomass. Quantitative analysis revealed that treatment at the MIC level resulted in a 66.55% decrease in biofilm mass compared to untreated controls (Table 4). This substantial inhibition indicates that LRML effectively disrupts biofilm architecture, possibly

Table 4 Anti-Biofilm Activity of Liquorice Extracts and LRML Against S. mutans

Treatment Group	Mean Biofilm Biomass Inhibition (%)	Standard Deviation (%)
Control	100	5.3
Liquorice Extract MIC	79	3.2
Liquorice Extract MBC	50.21	3.5
LRML MIC	66.55	2.1
LRML MBC	43.47	2.85
Liquorice Powder MIC	57.89	3.45
Liquorice Powder MBC	34.55	2.25

Abbreviations: MIC, Minimum Inhibitory Concentration; MBC, Minimum Bactericidal Concentration.

through interference with exopolysaccharide matrix synthesis or enhanced permeation of active constituents. The capacity to eradicate biofilms is of paramount importance in managing dental caries, as biofilm-associated S. mutans display elevated resistance to conventional antimicrobial therapy.

Discussion

The comprehensive preformulation and formulation evaluation of the LRML demonstrates its potential as an effective oral therapeutic agent. The UV-Visible spectrum of the licorice extract exhibited a distinct absorption maximum (λmax) at 277 nm, indicative of conjugated π-electron systems, primarily from flavonoids and glycyrrhizic acid derivatives. This observation aligns with previous studies, such as the one by Syeda Khair-ul-Bariyah et al, which reported similar λmax values for glycyrrhizic acid, confirming the presence of these compounds in licorice extracts.[22] The licorice extract demonstrated excellent suitability for quantitative analysis, as evidenced by a strong linear correlation (R^2 = 0.9809) between absorbance and concentration across the range of 5–100 µg/mL, conforming to the Beer-Lambert law. FTIR spectral analysis revealed characteristic absorption bands corresponding to key functional groups present in the bioactive constituents:[23] a broad peak at 3365 cm^{-1} indicated O-H stretching vibrations of hydroxyl groups in glycyrrhizin, while the sharp band at 1728 cm^{-1} represented C=O carbonyl stretching of the carboxylate moiety in glycyrrhizic acid. Notably, the spectra of the physical mixture (LRML) retained all significant absorption bands from both the drug and excipient without noticeable shifts or new peak formations, suggesting the absence of significant physicochemical interactions. This finding is consistent with studies that have utilized FTIR to confirm the compatibility of licorice components in various formulations.[24] DSC thermograms indicated a sharp endothermic peak at 95.77°C for pure licorice extract, corresponding to its melting point and suggesting a crystalline structure. Erythritol exhibited a distinct exothermic peak at 120.82°C, associated with its crystalline-to-amorphous transition. The physical mixture (LRML) displayed a single endothermic peak at 97.00°C, closely aligned with the melting point of licorice extract, indicating no interaction or degradation during mixing.[25] These thermal behaviors are in agreement with prior studies evaluating the thermal stability of licorice formulations.

The formulations demonstrated acceptable physicochemical properties, with formulation LRML-9 exhibiting the highest hardness value of 12.07 ± 0.22 kg/cm², attributed to its higher polymer concentration, particularly 2% w/w hydroxypropyl methylcellulose (HPMC). Drug content uniformity ranged between 51.22% and 96.00%, with standard deviations within acceptable limits, indicating consistent drug distribution. Residual moisture content remained below 1.5% across all formulations, suggesting adequate desiccation and stability.[26] Dissolution studies revealed that LRML-7, containing 1.2% HPMC and 1.5% methylcellulose, achieved a cumulative drug release of 96.87% within 45 minutes. This rapid release profile is likely due to the specific polymer blend facilitating efficient matrix hydration and erosion. Mathematical modeling confirmed the significant influence of composition variables on release kinetics, with a high predictive accuracy (R^2=0.98). Accelerated stability testing over three months demonstrated minimal changes in tablet hardness and drug release efficiency, indicating robust mechanical stability and sustained therapeutic performance. These results suggest that the LRML formulation maintains its integrity under stress conditions, supporting its potential for long-term storage.

The antimicrobial evaluation revealed distinct pharmacodynamic profiles for the crude licorice extract versus the optimized LRML formulation. While the unformulated extract demonstrated moderate activity against *S. mutans* (MIC = 12.5 µg/mL; MBC = 25 µg/mL), the LRML lollipop exhibited a remarkable 62.5-fold enhancement in potency (MIC/MBC=0.2 µg/mL). This dramatic improvement likely stems from multiple formulation advantages: (1) increased mucosal retention time enabling sustained drug release, (2) polymer-mediated stabilization of bioactive constituents against salivary degradation, and (3) synergistic penetration enhancement through citric acid-mediated pH modulation of the biofilm microenvironment.[27,28] Time-kill kinetics demonstrated concentration-dependent bactericidal action, with 4× MIC LRML achieving ≥3 $\log_{10}$ CFU/mL reduction within 8 hours a critical threshold for clinical efficacy in oral applications (CLSI, 2023). The rapid bactericidal effect (exceeding conventional chlorhexidine benchmarks at equivalent concentrations) suggests disruption of both membrane integrity (via glycyrrhizin's surfactant-like action) and intracellular targets (resveratrol-mediated inhibition of Gtf enzymes). These findings align with emerging paradigms in localized antimicrobial delivery, where formulation science can overcome the bioavailability limitations of phytochemicals while

minimizing resistance development through multi-target mechanisms.[29,30] The LRML formulation significantly inhibited biofilm formation by *S. mutans*, with a 66.55% reduction in total biomass at MIC levels. This substantial inhibition indicates that LRML effectively disrupts biofilm architecture, possibly through interference with exopolysaccharide matrix synthesis or enhanced permeation of active constituents.[31] The capacity to eradicate biofilms is paramount in managing dental caries, as biofilm-associated *S. mutans* display elevated resistance to conventional antimicrobial therapy. While the current study demonstrates the antimicrobial and anti-biofilm efficacy of the LRML formulation against *S. mutans*, it is limited by the use of a single-species model. Dental caries is driven by complex polymicrobial biofilms involving multiple bacterial taxa. Therefore, the findings may not fully capture the formulation's effectiveness in clinically relevant biofilm environments. Additionally, cytotoxicity assessments were not conducted in this study. Future investigations will focus on evaluating the biocompatibility of the formulation using oral epithelial or fibroblast cell lines, as well as its efficacy against a broader spectrum of oral pathogens, including *Lactobacillus* spp., *Actinomyces* spp., and *Candida albicans* to more accurately reflect the ecological diversity of dental plaque. Furthermore, to advance the clinical translation of LRML, future research should address formulation scalability, including optimization of manufacturing processes to ensure consistent quality and cost-effectiveness. Consideration of regulatory pathways, such as compliance with oral care product guidelines by agencies like the FDA or EMA, will be critical for eventual commercialization. Cost analysis comparing LRML to current commercial alternatives will also be valuable to assess its feasibility as an accessible preventive strategy, especially in resource-limited settings.

Conclusion

The comprehensive evaluation of the LRML formulation demonstrates its potential as an effective oral therapeutic agent for managing dental caries. Pre-formulation studies confirmed the chemical integrity and compatibility of licorice extract with pharmaceutical excipients. The formulation exhibited favorable physicochemical characteristics, rapid and efficient drug release, and robust stability. Moreover, its significantly enhanced antimicrobial and anti-biofilm activity against *S. mutans* highlights its promise in targeting cariogenic bacteria. Comparative analysis with existing literature, including studies conducted in China and India, reinforces the therapeutic relevance of this phytochemical-based delivery system. However, to further validate its clinical applicability, future research should include cytotoxicity studies on oral mucosal cells, broader antimicrobial testing against polymicrobial oral biofilms, and in-vivo or clinical evaluations to assess safety, efficacy, and patient acceptability of the LRML formulation.

Data Sharing Statement

The data and materials used in this study are available upon request. Researchers interested in accessing the dataset or related materials for academic and non-commercial purposes can contact the corresponding author prof Feiqui Wen (fwen62@163.com) for further information.

Ethics Approval

Present study approved form Sant Gajanan Maharaj College of Pharmacy, Mahagaon, site Chinchewadi, India.

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work. Abhinandan Ravsaheb Patil and Feiqiu Wen contributed equally to the last authorship.

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Disclosure

The authors declare no competing interests.

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