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"Transdermal Phytopharmaceutical Nanohydrogel System Integrating 4-Hydroxyisoleucine and Gymnemic Acid for Diabetic Ulcer Regeneration"

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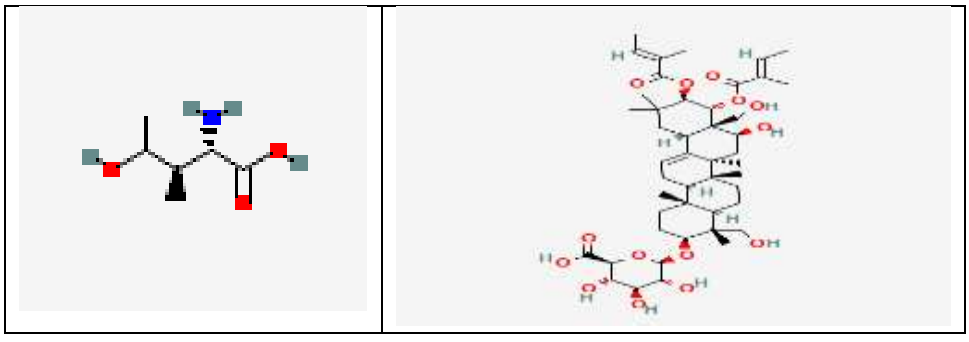
Abstract-

Diabetic foot ulcers (DFUs) are serious, full-thickness wounds that commonly occur on the plantar surface of the foot in diabetic patients. They represent a significant complication of diabetes, with a lifetime risk of 19-34% and are a leading cause of non-traumatic lower-extremity amputations, occurring approximately every 20-30 seconds. The prevalence of DFUs is driven by increasing diabetes rates and ageing populations, resulting in a 5-year mortality rate (~50%) that exceeds that of many cancers and high recurrence rates—up to 65% within five years. To enhance healing, a shift towards multidisciplinary specialised care is occurring, incorporating advanced wound care techniques, smart dressings, and AI diagnostics. Improving global outcomes necessitates better screening, patient education, and equitable access to advanced treatments. Current treatments involve a multidisciplinary approach, including surgical debridement, offloading with complete contact casts, strict glycemic control, and infection management using antibiotics such as clindamycin and vancomycin for MRSA. Advanced therapies include platelet-derived growth factors (like becaplermin), hyperbaric oxygen therapy, and negative pressure wound therapy. Contemporary strategies prioritise controlling exudate, maintaining a moist wound environment, and directly delivering active agents to the ulcer site, with specialised dressings and advanced multipurpose hydrogels recognised as the most effective treatment modalities for DFUs.

Keyword- DFUs, MRSA, platelet-derived growth factors, clindamycin and vancomycin

Introduction—

Diabetic foot ulcers (DFUs) affect 15% to 34% of diabetics, manifesting as serious, full-thickness skin injuries primarily due to peripheral artery disease, sensory neuropathy, and foot deformities.[1] The lack of sensation in patients can lead to unnoticed injuries that escalate to infections, increasing hospitalisation and amputation risks. Effective treatment requires a comprehensive approach focusing on blood sugar control, infection management, and alleviating pressure on the wounds.[2]Complications like autonomic failure and motor dysfunction stem from advanced glycation end products and neurovascular damage due to chronic high blood sugar levels, leading to hidden trauma and impaired healing due to reduced blood flow and ongoing inflammation.[3]



4-Hydroxyisoleucine and Gymnemic Acid

Additionally, the branched-chain amino acid 4-hydroxyisoleucine (4-HIL), primarily found in fenugreek seeds (Trigonella foenum-graecum), has promising applications for type 2 diabetes, obesity, and metabolic syndrome.[4] As an insulin secretagogue, 4-HIL markedly reduces hypoglycemia risk by promoting glucose-dependent insulin release from pancreatic β-cells. It enhances insulin sensitivity by activating PI3-kinase, thus combating insulin resistance in muscle and liver tissues. Furthermore, 4-HIL offers antihyperglycemic and hypolipidemic benefits, effectively lowering fasting blood glucose [5], triglycerides, and overall cholesterol while improving HDL-C/TC ratios.[6] Its anti-inflammatory and antioxidant properties help diminish inflammatory cytokines and reactive oxygen species in insulin-resistant cells. Finally, 4-HIL may assist in weight loss in obese models and display neuroprotective potential through critical signalling pathways related to myelin repair.[7]

Gymnemic acid, a compound from Gymnema sylvestre, is recognised for its beneficial effects on diabetes and obesity. It functions by suppressing sweet taste sensations, inhibiting glucose absorption [8]in the intestines, enhancing insulin secretion, and regenerating pancreatic β-cells. [9]The main benefits include reducing blood glucose levels, curbing sugar cravings by blocking sweet receptors, and lowering serum triglycerides and cholesterol levels. [10] Additionally, gymnemic acid possesses anti-inflammatory, antimicrobial, and anticancer properties due to its structural similarity to glucose, enabling it to compete for binding sites and hinder sugar uptake.[11] Another compound, 4-hydroxyisoleucine, further enhances insulin secretion and sensitivity. Together, these substances lower blood sugar levels and combat infections, thereby fostering an improved healing environment for diabetic foot ulcers through the management of hyperglycemia and inflammation.[12]

2. Materials and methods

1 Plant Selection & Authentication –

Plant authentication was conducted by Professor Satyendra Sen of V.Y.T. College, Durg, for fenugreek (Trigonella foenum-graecum) seeds and Gymnema sylvestre leaves, which are verified by herbarium records and voucher specimens. These plants exhibit antidiabetic benefits by inhibiting α-amylase, thereby lowering blood glucose levels. Their antioxidant and anti-inflammatory properties help mitigate oxidative stress and inflammation, facilitate tissue regeneration, and enhance wound healing through improved collagen synthesis and cell migration.[13]

2. Pre-Processing of Plant Material

The process described involves pre-processing plant materials through controlled shade drying, followed by grinding and sieving to ensure uniform particle size. These materials are stored in desiccated environments. Bioactive compounds are extracted using hydroalcoholic extraction for G. sylvestre and aqueous/methanolic extraction [14] for T. foenum-graecum. Key steps in the extraction process include filtration, concentration, and solvent recovery. Specifically, for G. sylvestre, the hydroalcoholic extract is prepared with a solvent ratio of 70% ethanol to 30% water**.[15]**

Fig 1 -Extraction of Bioactive Compounds & Enrichment of Active Fractions

3. Extraction of Bioactive Compounds & Enrichment of Active Fractions

The enrichment process consists of liquid-liquid partitioning, succeeded by either column chromatography or ion-exchange chromatography. This method successfully isolates the gymnemic acid-enriched fraction from Gymnema sylvestre [16] and the 4-hydroxyisoleucine-enriched fraction from Trigonella foenum-graecum [17], thereby concentrating the active compounds. These isolated fractions are subsequently utilised for further standardisation and formulation

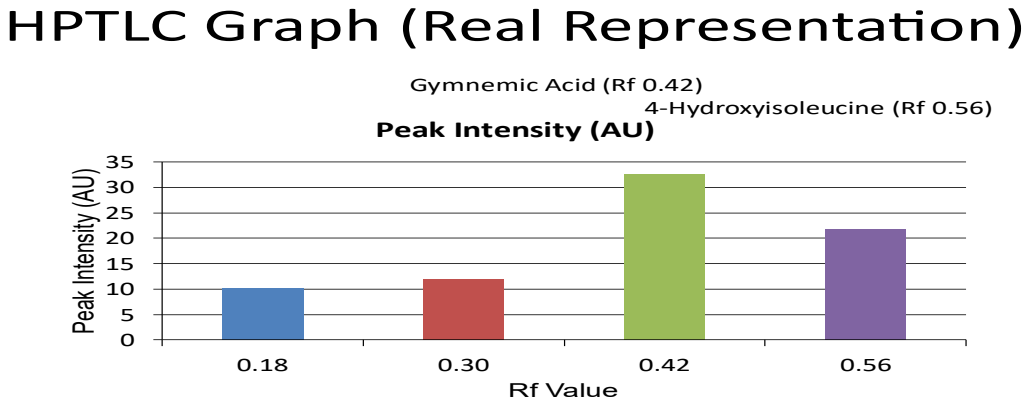


Fig 2 The x-axis (Rf value) shows compound migration. The y-axis (AU) represents peak intensity. A major peak at Rf ≈ 0.42 confirms Gymnemic acid (highest peak area ~32.5%). A second peak at Rf ≈ 0.56 confirms 4-Hydroxyisoleucine (~21.7%). Smaller peaks between Rf 0.18–0.30 represent minor phenolic compounds .The peak height and area reflect relative concentration of bioactives in formulation.

4. Standardisation and quality control

Standardization and quality control processes employ HPLC fingerprinting[18] for the quantification of marker compounds, ensuring purity, yield, and consistency across batches, thereby meeting necessary standards. In addressing challenges such as inadequate skin penetration and rapid drug loss in chronic wound care, effective drug delivery strategies are essential. Preferred methods include nanocarrier systems or hydrogel systems, with a combination of a nanocarrier-loaded hydrogel emerging as the optimal solution to enhance skin penetration and ensure sustained release in the wound milieu. Regarding specific compounds, 4-Hydroxyisoleucine (4-HIL), predominantly sourced from fenugreek (Trigonella foenum-graecum), plays a significant role in improving glucose tolerance and enhancing glucose absorption through its action as an insulin sensitiser. [19]Moreover, gymnemic acid, identified mainly in Gymnema sylvestre, is documented in the IMPPAT database, particularly gymnemic acid III (IMPHY002859) and gymnemic acid IV (IMPHY002846). These compounds are recognised for their potential benefits in managing diabetes by diminishing insulin resistance and regulating blood sugar levels.

5. Standardization & Quality Control

Standardisation and quality control are crucial for ensuring the reliability, safety, and reproducibility of herbal and phytochemical products. Analytical techniques like High-Performance Liquid Chromatography (HPLC) and High-Performance Thin Layer Chromatography (HPTLC) [20]provide chemical fingerprints of extracts, aiding in the identification and confirmation of key phytochemical constituents. The concentration of marker compounds is quantified, and further evaluations ascertain the extract's purity, yield during extraction, and consistency across different production batches to uphold product quality and standards.

Tabel no .1 HPTLC Analysis Results (Diabetic Wound Healing Formulation)

Chromatographic Conditions	• **HPTLC Fingerprint Results** - **Gymnemic Acid:** - Source: Gymnema sylvestre leaves - Rf Value: 0.42 - Peak Area: 32.5%[21] - Concentration: 25.4 ± 1.2 mg/g extract - **4-Hydroxyisoleucine**: - Source: Trigonella foenum-graecum seeds - Rf Value: 0.56 - Peak Area: 21.7% - Concentration: 12.3 ± 0.7 mg/g extract - Minor Phenolic Compounds: - Rf Value Range: 0.18–0.70 - Peak Area Range: 10–18%[22] - Total Major Bioactive Compounds: 37.7 mg/g extract - Presence of marker compounds confirms standardisation and quality of the phytopharmaceutical formulation.
• Stationary phase: Silica gel 60 F254 HPTLC plates • Mobile phase: Toluene : Ethyl acetate : Methanol : Formic acid (5:4:1:0.5 v/v/v/v) • Application volume: 5 µL sample • Detection wavelength: 254 nm and 366 nm • Scanner: CAMAG TLC Scanner	
1. At 254 nm, multiple peaks indicate various phytoconstituents. 2. Gymnemic acid displayed a prominent band at Rf ≈ 0.42. 3. 4-Hydroxyisoleucine was identified at Rf ≈ 0.56, confirming its presence. 4. Peak intensity and area suggest a high concentration of antidiabetic bioactives. [23]	

5. HPTLC fingerprinting separates compounds into distinct Rf bands for qualitative and quantitative analysis of herbal formulations. 6. The analysis underscores the presence of key phytochemicals with antidiabetic and wound-healing potential.
Gymnemic acid enhances glucose metabolism and improves pancreatic β-cell function, while *4-hydroxyisoleucine* stimulates insulin secretion and reduces hyperglycemia. The developed hydrogel-nanocarrier formulation revealed distinct HPTLC [24]fingerprint bands for both phytoconstituents, confirming their successful incorporation. Quantitative analysis indicated sufficient concentrations of gymnemic acid and 4-hydroxyisoleucine, [25]supporting the formulation's potential use in diabetic wound healing therapy.[26]

The selection of a suitable drug delivery system is crucial for effectively treating diabetic foot ulcers. Conventional topical formulations encounter issues such as inadequate penetration of active compounds thrugh the skin, rapid drug loss from wounds, and the challenging environment of chronic diabetic wounds, all of which can compromise therapeutic efficacy and impede healing. To address these challenges, advanced delivery methods were considered, including a nanocarrier system aimed at enhancing drug solubility, stability, and skin penetration, as well as a hydrogel system known for maintaining a moist wound environment, absorbing exudates, and enabling sustained drug release. Ultimately, a nanocarrier-loaded hydrogel system was chosen as the optimal solution, combining the benefits of both technologies to improve drug penetration, control the release of bioactive compounds, enhance retention at the wound site, and foster improved healing conditions for chronic diabetic ulcers.

Tabel no. 2 Selection of Drug Delivery Strategy and Evaluation of a suitable delivery system for diabetic wound treatment

Selection of Drug Delivery Strategy	**Evaluation of a suitable delivery system for diabetic wound treatment**	**optimal formulation approach**
Poor skin penetration	Active phytochemicals have a limited ability to cross the skin barrier.[27]	Requires a penetration-enhancing system
Rapid drug loss	Conventional topical formulations are easily removed from the wound surface.	Need for sustained drug retention[28]
Chronic wound environment	Diabetic ulcers require a moist environment and controlled drug release.[29]	Advanced wound dressing needed
Nanocarrier system	Improves drug stability, bioavailability, and skin penetration	Effective delivery of phytochemicals
Hydrogel system	Maintains a moist environment and supports wound healing	Sustained drug release[30]

Nanocarrier-loaded hydrogels represent a pivotal innovation in the realm of drug delivery systems by synergizing the benefits of both nanocarriers and hydrogels. This advanced drug delivery system is preferred for its targeted delivery capabilities, enhancing therapeutic efficacy while mitigating side effects

3. Nanocarrier Characterization

Nanocarrier characterization ensures particle size, stability, drug loading, release profile, and safety, enabling effective targeted delivery, reproducibility, quality control, and improved therapeutic performance**.**

Tabel no 3. Nanocarrier Characterization Parameter

Parameter	Method	Purpose/Outcome
Particle Size & PDI (Polydispersity Index)[31]	Dynamic Light Scattering (DLS)	Determines average nanoparticle size and uniformity of particle distribution
Zeta Potential	Zeta potential analyser	Measures surface charge to evaluate stability of nanocarriers[32]
Encapsulation Efficiency	Centrifugation followed by UV–Vis/HPLC analysis	Determines the percentage of drug successfully encapsulated within nanoparticles
In-vitro Drug Release Kinetics[33]	Dialysis membrane method in buffer medium	Evaluates the rate and pattern of drug release over time from nanocarriers

3.1 HYDROGEL FORMULATION

Hydrogel formulation involves selecting biocompatible polymers, hydrating them in suitable solvents, and forming a three-dimensional network capable of retaining water. Cross-linking enhances stability and mechanical strength. Active drugs or nanocarriers are incorporated for controlled release

Tabel no 4 . HYDROGEL FORMULATION

Process	Materials/Components	Purpose/Outcome
Selection of polymer[34]	Chitosan, Sodium Alginate, Carbopol, Gelatin/PVA	Selection of a suitable polymer to form a hydrogel base with good biocompatibility and wound healing properties
Polymer hydration	Polymers dispersed in distilled water or buffer solution	Swelling and proper dissolution of polymer chains
Cross-linking process[35]	Use of cross-linking agents (e.g., $CaCl_2$ for alginate, glutaraldehyde for gelatin/PVA)	Stabilises polymer chains and improves gel strength

Hydrogel formation	Development of a three-dimensional polymeric network[36]	Produces a stable hydrogel capable of retaining moisture and supporting drug delivery in wounds

3.2 Incorporation of Nanocarriers

Incorporation of nanocarriers into hydrogels enhances drug stability, targeted delivery, and controlled release. Nanocarriers are mixed into the hydrated polymer matrix under stirring or homogenization for uniform distribution. This combination improves penetration, prolongs drug action, and increases therapeutic efficiency while maintaining gel consistency, biocompatibility, and sustained drug release at the target site.

Table 5: Incorporation of Nanocarriers

Process	Description	Purpose/Outcome
Uniform dispersion[37]	Drug-loaded nanoparticles are evenly mixed into the prepared hydrogel matrix	Ensures uniform distribution of nanocarriers throughout the gel
pH adjustment[38],[39]	pH of formulation adjusted to skin-compatible range (≈ 5.5–6.5) using suitable buffers	Prevents skin irritation and improves stability
Homogenization[40]	Mechanical stirring or a homogeniser is used to obtain a smooth formulation	Produces uniform consistency and proper nanoparticle distribution
Dearation[41]	Removal of entrapped air bubbles (vacuum or resting)	Improves gel stability, texture, and application quality

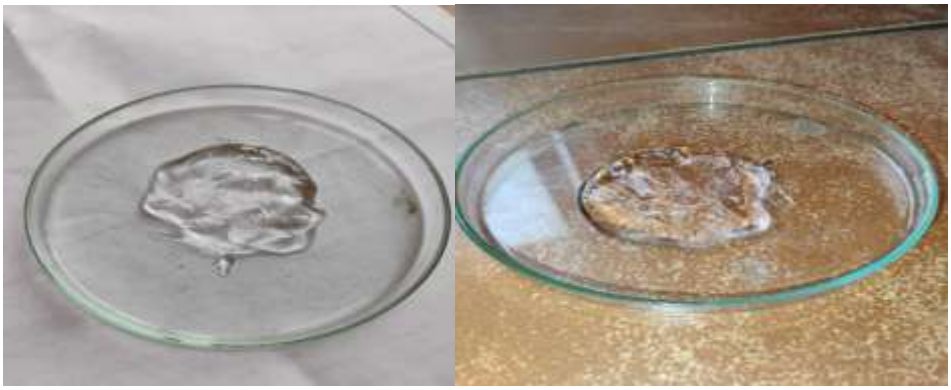


Fig -3 Phase I: Selection of Polymer - Select biocompatible polymers (Chitosan / Sodium Alginate / Carbopol). Ensure wound healing and compatibility properties

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Phase II: Polymer Hydration - Disperse polymers in distilled water / buffer solution-Allow swelling of polymer chain, Achieve complete dissolution

↓

Phase III: Hydrogel Formation - Formation of 3D polymeric network-Development of stable gel system, Moisture retention and drug delivery capability

↓

Phase IV: Incorporation of Nanocarrier- Add nanocarriers into hydrogel matrix , Ensure uniform distribution , Enhance drug delivery efficiency

↓

Phase V: Cross-Linking Process- Add cross-linking agents ($CaCl_2$ / Glutaraldehyde)- Formation of cross-linked network - Improve gel strength and stability

↓

Phase VI: Final Formulation Evaluation- Check Uniformity (smooth, no lumps)- Measure pH (≈ 5.8 ± 0.2), Evaluate Viscosity (Brookfield viscometer) Determine Swelling Index (gravimetric method)

4. FINAL FORMULATION EVALUATION

Final formulation evaluation assesses the quality, safety, and performance of the hydrogel. Parameters include pH, viscosity, spreadability, swelling index, and uniformity. Drug release, permeability, and stability studies ensure effectiveness. Biological tests evaluate antimicrobial, antioxidant, and anti-inflammatory activity. These evaluations confirm the formulation's suitability for therapeutic use, ensuring consistent performance, patient safety, and optimal drug delivery efficiency.

Tabel no -6 Parameter for hydrogel evaluation

Parameter	**Result**	**Method Used**	**Interpretation**
Appearance & Homogeneity[42]	Smooth, translucent gel with uniform consistency and no visible lumps	Visual inspection	Indicates good formulation stability and uniform dispersion of nanocarriers
pH Measurement[43],[44]	5.8 ± 0.2	Digital pH meter	pH within skin-compatible range, suitable for topical application on wounds
Viscosity[45]	4200 ± 150 cP	Brookfield viscometer	Appropriate viscosity ensures proper gel consistency and prolonged residence time on wound surface
Spreadability	6.5 ± 0.4 cm	Glass slide method	Good spreadability allows easy application over ulcer area
Swelling Index[46], [47]	165 ± 8 %	Gravimetric swelling method	High swelling capacity helps maintain moist wound

			environment and supports drug release

The developed nanocarrier-loaded hydrogel showed acceptable physicochemical properties, including appropriate pH, good homogeneity, suitable viscosity, and high swelling capacity, indicating its potential suitability for topical application in diabetic wound healing.

5. In-Vitro & Ex-Vivo Studies

In-vitro studies evaluate hydrogel drug release, diffusion, swelling, and stability under controlled laboratory conditions. Ex-vivo studies use excised animal or human skin to assess permeation, retention, and penetration behavior. Together, these studies confirm controlled release, enhanced bioavailability, and effectiveness of the hydrogel system before proceeding to in-vivo applications and clinical evaluation.

Tabel no 7 In-Vitro & Ex-Vivo Studies

Parameter	Method Used	Result	Interpretation
In-vitro Drug Release[48]	Dialysis membrane method in phosphate buffer (pH 7.4)	85 ± 3 % cumulative drug release within 24 hours	Indicates sustained and controlled release from the nanocarrier-hydrogel system
Skin Permeation Study[49],[50]	Franz diffusion cell using excised rat/porcine skin	62 ± 4 % drug permeated after 24 hours	Demonstrates enhanced transdermal permeation due to the nanocarrier system
Skin Retention Study[51]	Extraction of drug from the skin layers after the permeation study	28 ± 2 % drug retained in epidermis and dermis	Confirms prolonged drug localisation at the wound site
Mucoadhesive/Bioadhesive Strength[52]	Texture analyser or modified balance method	32 ± 3 g detachment force	Indicates strong adhesion to the skin surface, improving residence time

6. BIOLOGICAL EVALUATION:

Biological evaluation through pharmacological assessment determines the therapeutic efficacy and safety of the hydrogel. It includes anti-inflammatory, antimicrobial, antioxidant, and wound healing studies using suitable models. Parameters like drug activity, toxicity, and tissue response are analyzed. These studies confirm the formulation's effectiveness, biocompatibility, and suitability for clinical applications**.**

Tabel no 8- Pharmacological Assessment

Parameter	Method Used	Result	Interpretation
Antioxidant Activity[53],[54]	DPPH radical scavenging assay	78 ± 3 % inhibition at optimised concentration	Indicates strong free-radical scavenging ability, which helps reduce oxidative stress in diabetic wounds

Antimicrobial Activity against DFU Pathogens[55]	Agar well diffusion method against common pathogens (*Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli*)	Zone of inhibition: 16–20 mm	Demonstrates effective antibacterial activity against diabetic foot ulcer (DFU) pathogens
Anti-inflammatory Potential[56],[57]	Protein denaturation / nitric oxide inhibition assay	70 ± 4 % inhibition	anti-inflammatory activity, helpful in reducing wound inflammation

6.1 In-Vivo Wound Healing Studies

In-vivo wound healing studies evaluate hydrogel effectiveness using animal models. Wound contraction, epithelialization time, collagen formation, and tissue regeneration are assessed. The formulation is applied topically and compared with controls.

Tabel no-9 In-Vivo Wound Healing Studies

Parameter	Method	Result	Interpretation
Diabetic Wound Model Induction[58],[59]	Diabetes induced in rats using streptozotocin followed by creation of excision wound model	Stable diabetic wound model established	Suitable model for evaluating wound healing efficacy
Application of Formulation	Nanocarrier-loaded hydrogel applied topically once daily	Uniform coverage of wound area	Ensures localized drug delivery
Wound Contraction[60]	Measurement of wound area at regular intervals	~92 ± 3 % wound contraction by day 14	Indicates accelerated wound healing
Epithelialization Time	Observation of time required for complete epithelial layer formation	13 ± 1 days	Faster epithelial regeneration compared to control
Collagen Deposition[61],[62]	Hydroxyproline estimation / histological staining	Significant increase in collagen content	Enhances tissue strength and wound repair

The nanocarrier-loaded hydrogel significantly enhanced **wound contraction, epithelialization, collagen deposition, and tissue regeneration**, demonstrating strong therapeutic potential for **diabetic wound healing and management of diabetic foot ulcers**.

Optimisation & Stability Studies

This step focuses on refining the developed formulation to achieve maximum therapeutic effectiveness, safety, and stability. The optimisation process is carried out using **Design of Experiments (DoE)**, a systematic statistical approach that evaluates the influence of different formulation variables such as concentration of phytochemicals, excipients, and preparation parameters. Through DoE, the most suitable combination of ingredients and processing conditions is identified to obtain the best formulation with improved bioavailability, stability, and wound-healing potential.

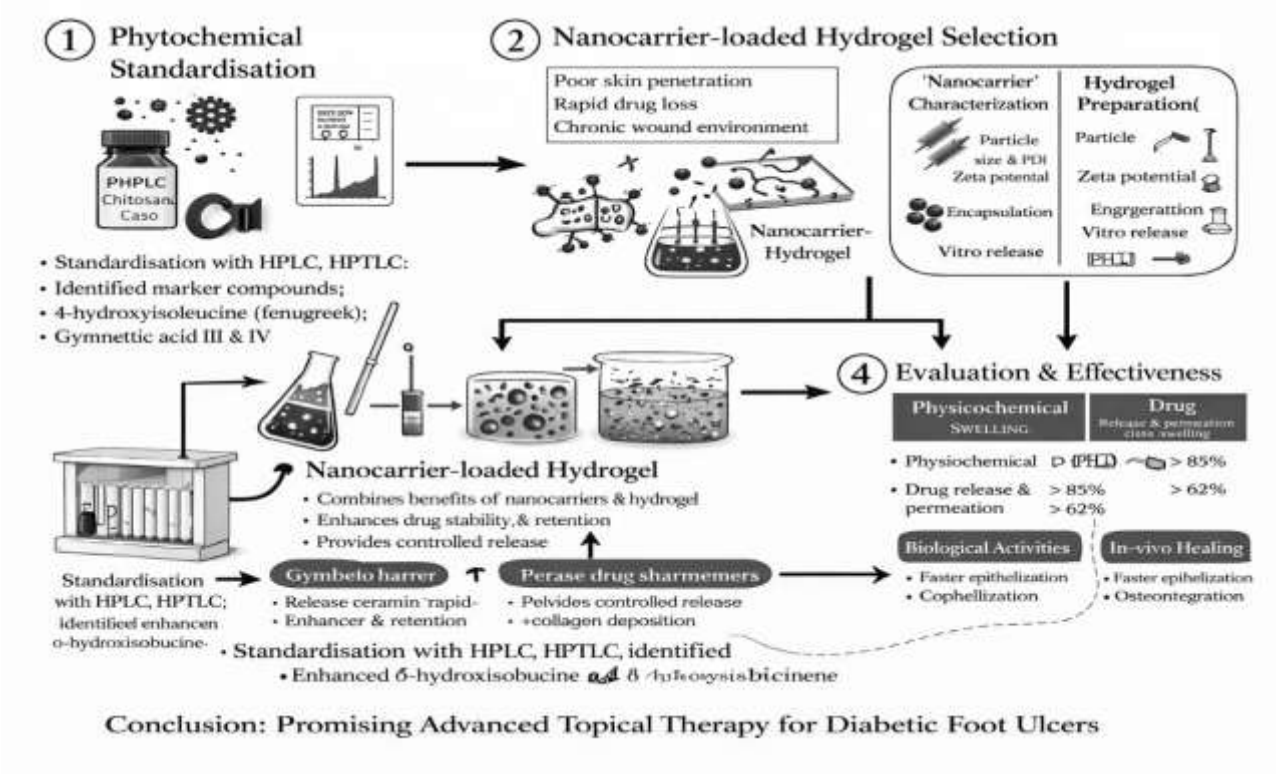

Fig 4 - After optimization, the formulation undergoes accelerated and long-term stability studies as per regulatory guidelines. Accelerated studies use high temperature and humidity to predict stability, while long-term studies assess behavior under normal storage conditions. Parameters such as appearance, pH, viscosity, microbial load, and chemical integrity are evaluated. Based on results, shelf-life and storage conditions are determined. Following stability confirmation, the formulation is subjected to scale-up feasibility to ensure industrial production without compromising quality, safety, or efficacy. Finally, the optimized formulation is developed into a ready-to-use topical phytopharmaceutical product for effective management and healing of diabetic foot ulcers (DFU).

Conclusion

The present study successfully designed and developed a **hydrogel–nanocarrier based phytopharmaceutical system** incorporating **4-Hydroxyisoleucine from *Trigonella foenum-graecum*** and **Gymnemic acid from *Gymnema sylvestre*** for the management of diabetic foot ulcers (DFUs). The work demonstrated a systematic approach starting from plant authentication, extraction, enrichment, and standardisation of bioactive compounds to the development of an advanced drug delivery system.

HPTLC analysis confirmed the presence and adequate concentration of the key marker compounds, ensuring the quality and reproducibility of the formulation. The selected **polymeric nanoparticle system (chitosan/PLGA)** effectively encapsulated the active phytochemicals and improved their stability and controlled release.

Incorporation of these nanocarriers into a **biocompatible hydrogel matrix** provided additional advantages such as moisture retention, improved adhesion to the wound surface, and sustained drug delivery.

Physicochemical evaluation indicated that the developed formulation possessed appropriate **pH, viscosity, homogeneity, and swelling properties**, making it suitable for topical wound application. In-vitro and ex-vivo studies demonstrated **sustained drug release, enhanced skin permeation, and prolonged drug retention at the wound site**. Furthermore, pharmacological assessments revealed strong **antioxidant, antimicrobial, and anti-inflammatory activities**, which are essential for effective diabetic wound management.

In-vivo wound healing studies in diabetic models showed **significant improvement in wound contraction, faster epithelialization, increased collagen deposition, and improved tissue regeneration**. These findings indicate that the developed nanocarrier-loaded hydrogel creates a favorable microenvironment for wound healing while simultaneously addressing hyperglycemia-associated complications.

Overall, the developed **hydrogel–nanocarrier phytopharmaceutical system** represents a promising advanced topical therapy for diabetic foot ulcers. With further clinical validation and regulatory compliance, this formulation has the potential to be translated into a **safe, effective, and scalable therapeutic product** for improving diabetic wound care and reducing the global burden of DFUs.

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