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Quality by Design (QbD)-Based Development and Optimization of a Polyherbal Nanoformulation of Gymnema Sylvestre, Trigonella Foenum-Graecum and Momordica Charantia for Multitargeted Management of Type 2 Diabetes Mellitus

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ABSTRACT:

Type 2 diabetes mellitus (T2DM) is a multifactorial metabolic disorder characterized by insulin resistance, progressive pancreatic beta-cell dysfunction, persistent hyperglycemia, abnormal lipid metabolism, oxidative stress and an increased risk of microvascular and macrovascular complications. The present study was designed to develop and optimize a polyherbal nanoformulation containing standardized extracts of Gymnema sylvestre, Trigonella foenum-graecum and Momordica charantia using a Quality by Design (QbD) approach. The three herbs were selected because their phytoconstituents may act through complementary mechanisms including inhibition of intestinal carbohydrate-digesting enzymes, improvement of glucose utilization, modulation of insulin secretion/sensitivity and attenuation of oxidative stress. Hydroalcoholic extracts were standardized using gymnemic acids, 4-hydroxyisoleucine and charantin as respective phytochemical markers. Chitosan-tripolyphosphate nanoparticles were prepared using ionic gelation. A Quality Target Product Profile (QTPP) and critical quality attributes (CQAs) were established, followed by risk assessment. Chitosan concentration (X1), sodium tripolyphosphate concentration (X2) and sonication time (X3) were optimized using a three-factor Box-

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Behnken design. Particle size, polydispersity index and entrapment efficiency were considered primary responses. In the illustrative optimized dataset, the nanoformulation exhibited a mean particle size of approximately 181 nm, PDI of 0.212, positive zeta potential of +28.7 mV and overall marker entrapment efficiency of 82.4%. A biphasic and sustained phytoconstituent release pattern was observed over 24 h. The optimized nanoformulation showed greater inhibitory activity against alpha-glucosidase and alpha-amylase than the unencapsulated polyherbal blend. In an illustrative high-fat-diet/streptozotocin-induced T2DM model, the nanoformulation produced progressive reductions in fasting blood glucose and improvements in HbA1c, insulin resistance and lipid parameters relative to untreated diabetic controls. QbD-based optimization provided a systematic relationship between formulation/process variables and nanoparticle performance and established a preliminary design space and control strategy. These findings support further experimental investigation of standardized polyherbal chitosan nanoparticles as a multitarget phytopharmaceutical delivery platform. However, pharmacokinetic studies, long-term toxicological assessment, batch-to-batch botanical standardization and appropriately designed clinical trials are required before therapeutic conclusions can be made.

1. INTRODUCTION

Type 2 diabetes mellitus is a chronic and progressive metabolic disorder in which impaired insulin action is accompanied by progressive failure of pancreatic beta-cells to maintain adequate insulin secretion. Persistent hyperglycemia is associated with dyslipidemia, oxidative stress, inflammatory abnormalities and progressive damage to the cardiovascular, renal, neural and ocular systems. Diabetes represents an increasingly important global health burden. The International Diabetes Federation estimated that approximately 589 million adults aged 20-79 years were living with diabetes in 2024 and projects an increase to approximately 853 million by 2050; more than 90% of cases are type 2 diabetes. [1]

The multifactorial nature of T2DM has generated interest in therapeutic strategies capable of simultaneously influencing intestinal glucose absorption, insulin secretion, insulin sensitivity, hepatic glucose production, oxidative stress and lipid metabolism. Medicinal plants represent one potential source of chemically diverse compounds with such complementary biological activities. Gymnema sylvestre contains triterpenoid saponins collectively referred to as gymnemic acids and has been investigated for effects on carbohydrate absorption, insulin secretion and glucose homeostasis. A systematic review and meta-analysis of ten studies involving 419 participants reported improvements in several glycemic parameters following G. sylvestre supplementation, although substantial heterogeneity was present in the available evidence. [2-4]

Trigonella foenum-graecum (fenugreek) contains 4-hydroxyisoleucine, trigonelline, steroidal saponins and soluble galactomannans. Meta-analytic evidence suggests that fenugreek may improve fasting glucose, postprandial glucose and HbA1c, although differences in preparations, dosage and study design complicate direct comparison among studies. Momordica charantia contains cucurbitane-type triterpenoids, saponins and several other compounds associated experimentally with alpha-glucosidase inhibition, modulation of insulin signaling and altered glucose metabolism. A 2024 meta-analysis of eight randomized trials involving 423 participants reported reductions in fasting glucose, postprandial glucose and HbA1c, but the authors emphasized the need for additional high-quality studies. [5-10]

Despite their pharmacological potential, botanical preparations present challenges including phytochemical variability, poor stability, variable intestinal permeability, low or inconsistent oral bioavailability and difficulties in reproducible manufacturing. Nanocarrier systems may provide opportunities to protect phytoconstituents, modify their release and improve formulation reproducibility. Chitosan-TPP nanoparticles are particularly attractive because they can be prepared by aqueous ionic gelation without harsh organic processing. Their properties, however, are highly sensitive to polymer concentration, molecular characteristics, solution environment and processing parameters. [11-18]

Quality by Design provides a systematic framework for addressing this complexity. ICH Q8 describes pharmaceutical development based on predefined objectives, product/process understanding, risk management and design of experiments, while ICH Q9 and Q10 integrate risk management and lifecycle quality systems. Accordingly, the present study proposes a

QbD-driven strategy for developing and optimizing a standardized three-herb chitosan nanoparticle system intended for multitarget experimental management of T2DM. [19-21]

2. MATERIALS AND METHODS

2.1 Materials, Collection and Authentication of Plant Materials

Leaves of Gymnema sylvestre R. Br., seeds of Trigonella foenum-graecum L. and immature fruits of Momordica charantia L. were selected as the botanical materials. Plant materials should be obtained from a certified herbal raw-material supplier or collected from an authenticated geographical source during the appropriate harvesting season. Authentication should be performed by a qualified botanist or pharmacognosist using macroscopic, microscopic and taxonomic characteristics. Voucher specimens of each plant should be deposited in a recognized departmental herbarium, and unique voucher numbers must be inserted in the final experimental manuscript. [22,23]

The plant materials should be inspected for foreign matter, fungal contamination and visible deterioration. Samples are washed when appropriate, shade-dried initially and subsequently dried under controlled conditions at approximately 40-45 degrees C until constant mass is obtained. Excessive temperature should be avoided to minimize degradation of thermolabile constituents. Dried materials are separately pulverized and passed through a suitable sieve, for example 40 mesh, to obtain uniform powders. The powders are stored in airtight light-resistant containers containing appropriate desiccant until extraction.

Low-molecular-weight chitosan of defined molecular weight and degree of deacetylation should be used as the cationic polymer. Sodium tripolyphosphate (TPP) is used as the ionic cross-linking agent. Glacial acetic acid, ethanol, methanol or acetonitrile of analytical/HPLC grade, purified water and other analytical reagents are used as required. Trehalose or mannitol may be evaluated as a cryoprotectant during lyophilization. Reference standards of gymnemic acid or a validated gymnemic-acid marker, 4-hydroxyisoleucine and charantin or an appropriate validated M. charantia marker are required for chromatographic standardization. Alpha-amylase, alpha-glucosidase, soluble starch, p-nitrophenyl-alpha-D-glucopyranoside, acarbose, metformin and reagents for biochemical estimations should be procured from reputable manufacturers. [4,7,9,15-18]

All reagents, equipment model numbers, supplier names, lot numbers and analytical-grade information should be recorded in the final study to strengthen traceability and reproducibility.

2.2 Preparation and Standardization of Herbal Extracts

The powdered botanical materials are extracted separately so that the identity, yield and phytochemical profile of each component can be controlled before preparation of the final polyherbal blend. For G. sylvestre leaves and M. charantia fruits, approximately 100 g of dried powder may be extracted with 70% ethanol using a solid-to-solvent ratio of approximately 1:10. Extraction can be performed by ultrasound-assisted extraction for 30-45 min followed by maceration or by three successive reflux cycles under temperature-controlled conditions. Fenugreek seeds may initially be defatted with petroleum ether when necessary and subsequently extracted using 70% ethanol to obtain polar and moderately polar phytoconstituents.

The pooled extracts are filtered and concentrated under reduced pressure using a rotary evaporator at temperatures not exceeding approximately 45 degrees C. The concentrated extracts are freeze-dried or vacuum-dried to obtain free-flowing powders. Percentage extractive yield is calculated as: Extractive yield (%) = [Weight of dried extract / Weight of starting plant powder] x 100.

Each dried extract should initially undergo phytochemical screening for major classes including alkaloids, flavonoids, phenolics, saponins, steroids/triterpenoids and carbohydrates. Quantitative chromatographic fingerprinting is then employed to provide more meaningful standardization. Gymnemic acids may be quantified in the G. sylvestre extract using a validated HPLC or HPTLC method. The fenugreek extract is standardized using 4-hydroxyisoleucine and/or trigonelline. The M. charantia extract is standardized using charantin or, preferably where analytical capability permits, a panel of characteristic cucurbitane-type triterpenoids. [4,7,9,10,22,23]

Chromatographic method validation should cover specificity, linearity, accuracy, precision, range, limit of detection and limit of quantification in accordance with applicable analytical-validation guidance. For development of the nanoformulation, standardized dried extracts are blended on an equal dry-solid basis (1:1:1) unless preliminary mixture-design studies support another ratio. Selection of the final ratio should consider marker content as well as alpha-amylase/alpha-glucosidase inhibitory activity. The blend is stored under moisture-protected conditions. This marker-based standardization is particularly important

because variability of botanical starting materials represents one of the major sources of variability in herbal product development. [24,25]

2.3 Definition of QTPP, Critical Quality Attributes and Risk Assessment

Development was initiated by defining the Quality Target Product Profile (QTPP), consistent with the principles of ICH Q8(R2). The intended product was an orally administered, lyophilized, redispersible chitosan-based polyherbal nanoparticle system capable of delivering standardized phytochemical markers from the three botanical extracts. Important target characteristics included nanoscale particle size, narrow size distribution, adequate marker entrapment, physical stability, reproducible phytochemical content and controlled release. [19-21]

Critical Quality Attributes (CQAs) were selected on the basis of their potential relationship with product performance and stability. Mean particle size was considered critical because it can influence colloidal stability, intestinal interaction and release characteristics. Polydispersity index was selected as an indicator of the uniformity of the nanoparticle population. Zeta potential was considered relevant to colloidal stability and the cationic nature of chitosan. Entrapment efficiency was important for reproducible delivery of standardized phytochemical markers. Additional CQAs included marker content, redispersibility after lyophilization, release profile, moisture content and physical appearance. [15-21]

Potential critical material attributes included chitosan molecular weight, degree of deacetylation, chitosan concentration, TPP concentration, extract loading, pH and phytochemical composition of the extract blend. Potential critical process parameters included mixing speed, order and rate of TPP addition, mixing duration, sonication time, sonication energy and temperature. A preliminary Ishikawa diagram should classify risks into material, method, machine, measurement, environment and formulation categories. Failure Mode and Effects Analysis (FMEA) may then be used to rank variables. Severity, occurrence and detectability can each be scored, and a risk-priority number calculated. Variables showing the strongest potential impact on particle size, PDI and entrapment efficiency are advanced to experimental optimization.

For the present model, chitosan concentration, TPP concentration and sonication time were identified as high-risk variables. Literature on chitosan-TPP nanoparticles shows that polymer concentration and formulation environment can substantially alter hydrodynamic particle size and polydispersity, supporting their inclusion as critical variables. [15-18]

Table 1. Proposed QTPP and CQAs for the polyherbal nanoformulation

QTPP/CQA	Target	Justification
Dosage form	Lyophilized/redispersible nanoparticles	Improved storage and handling
Route	Oral	Appropriate for chronic experimental administration
Particle size	100-250 nm	Nanoscale dispersion with acceptable uniformity
PDI	<=0.30	Narrow particle-size distribution
Zeta potential	>= +/-20 mV, preferably positive	Colloidal stability/chitosan surface
Entrapment efficiency	>=75%	Adequate loading of phytochemical markers
Marker assay	90-110% of target	Batch consistency
Release	Controlled/biphasic over 24 h	Protection followed by prolonged release
Redispersibility	No visible aggregation	Product usability
Stability	Minimal CQA change during storage	Reproducible quality

2.4 Experimental Design and QbD-Based Optimization

A three-factor, three-level Box-Behnken response-surface design was selected for optimization because it enables assessment of linear, quadratic and interaction effects with fewer experiments than a full three-level factorial design. Design of experiments has been increasingly applied to nanocarrier development because particle size, PDI and entrapment efficiency frequently depend on interacting rather than isolated formulation variables. [19-21]

The independent variables were chitosan concentration (X1), TPP concentration (X2) and sonication time (X3). Chitosan was evaluated from 0.15 to 0.35% w/v, TPP from 0.05 to 0.15% w/v and sonication time from 3 to 9 min. Seventeen experimental runs were generated, including five replicated center points to estimate pure experimental error. The principal responses were mean hydrodynamic particle size (Y1), polydispersity index (Y2) and overall phytochemical entrapment efficiency (Y3). Particle size and PDI were minimized, whereas entrapment efficiency was maximized.

The second-order polynomial model used was: $Y = beta0 + beta1X1 + beta2X2 + beta3X3 + beta12X1X2 + beta13X1X3 + beta23X2X3 + beta11X1^2 + beta22X2^2 + beta33X3^2$, where Y represents the response, beta0 the intercept, beta1-beta3 the linear coefficients, beta12-beta23 the interaction coefficients and beta11-beta33 the quadratic coefficients.

Analysis of variance is used to determine model significance, significant factors, coefficient of determination, adjusted R2 and lack-of-fit. Three-dimensional response surfaces and contour plots are generated to visualize factor interactions. A numerical desirability function integrates all CQAs. Target criteria may specify particle size around 150-200 nm, PDI close to or below 0.20-0.25 and entrapment efficiency greater than approximately 80%. A checkpoint batch is subsequently manufactured at the predicted optimum and observed values are compared with predicted values. A prediction error below approximately 5-10% is considered supportive of the mathematical model for preliminary development. [19-21]

2.5 Preparation of Polyherbal Chitosan Nanoparticles by Ionic Gelation

Polyherbal nanoparticles are prepared through ionotropic/ionic gelation between positively charged amino groups of chitosan and negatively charged phosphate groups of TPP. Ionic gelation is advantageous during botanical formulation development because nanoparticle formation can be achieved predominantly in an aqueous system without the extensive use of aggressive organic solvents. Chitosan-TPP ionic gelation has been extensively studied and can be statistically optimized to obtain nanoparticles with controlled dimensions. [15-18]

The required quantity of chitosan is dispersed in 0.5-1.0% v/v aqueous acetic acid under magnetic stirring and allowed to hydrate overnight. The pH is subsequently adjusted to approximately 5.0-5.5 using dilute sodium hydroxide. Maintaining pH is important because the degree of protonation of chitosan influences electrostatic interaction with TPP and nanoparticle formation. The standardized polyherbal extract blend is dissolved or dispersed in a minimal volume of aqueous hydroethanolic vehicle and added to the chitosan solution under continuous stirring. Residual organic solvent, where used, should be minimized before nanoparticle formation. [15,16,18]

TPP solution is freshly prepared in purified water and added dropwise to the drug-loaded chitosan solution at a controlled rate while stirring at approximately 700-1000 rpm. Nanoparticle formation occurs spontaneously through electrostatic crosslinking. The dispersion is then subjected to probe sonication at the selected experimental duration while controlling sample temperature using an ice bath. [15,18]

The nanoparticles are separated by high-speed refrigerated centrifugation or tangential/ultrafiltration. The pellet is washed with purified water to remove unentrapped extract and excess cross-linker. A cryoprotectant such as trehalose may be incorporated before freeze-drying. The lyophilized nanoparticles are stored in airtight, light-resistant containers under controlled conditions. Before characterization, a defined quantity is reconstituted in purified water and visually examined for dispersibility, sedimentation and aggregation. Process yield is calculated from the mass of recovered dried nanoparticles relative to the theoretical solids content. [18]

2.6 Physicochemical and Solid-State Characterization

Particle size and polydispersity index are determined using dynamic light scattering after appropriately diluting the nanoparticle dispersion in filtered purified water. Measurements should be performed in triplicate at approximately 25 degrees C, and both intensity distribution and Z-average diameter should be recorded. A PDI below approximately 0.30 is generally considered evidence of a relatively uniform nanoparticle dispersion for preliminary pharmaceutical-development purposes. [15-18]

Zeta potential is determined by electrophoretic light scattering. Because chitosan possesses protonatable amino groups, unloaded and extract-loaded particles are generally expected to display a positive surface charge when chitosan is available at the nanoparticle surface. Changes in zeta potential following phytochemical loading may provide indirect evidence of extract-polymer interaction. Entrapment efficiency is measured by separating free phytoconstituents from the nanoparticle-bound fraction through ultracentrifugation or an ultrafiltration membrane. Concentrations of the selected herbal marker compounds in the supernatant are determined chromatographically. Entrapment efficiency is calculated as EE (%) = [(Total marker - Free marker) / Total marker] x 100. Loading capacity is calculated using the quantity of marker entrapped per unit mass of recovered nanoparticles. [15-18]

Particle morphology may be studied using transmission electron microscopy or scanning electron microscopy after appropriate sample preparation. Nanoparticles are expected to show near-spherical morphology without extensive aggregation. Fourier-transform infrared spectroscopy may be performed on individual extracts, chitosan, TPP, physical mixtures and optimized nanoparticles to evaluate major chemical interactions. Differential scanning calorimetry and powder X-ray diffraction may be used to examine whether encapsulation changes the crystalline/amorphous characteristics of botanical marker constituents. [15-18]

Additional tests include percentage yield, moisture content, redispersibility index, pH, marker content uniformity and total phenolic/saponin retention where relevant. The combined characterization approach is required because particle size alone cannot establish nanoparticle quality; chemical standardization and phytochemical retention are equally important for a polyherbal formulation.

2.7 *In-Vitro* Release, Release Kinetics and Stability Assessment

In-vitro phytochemical release from the optimized nanoparticles is evaluated using a dialysis-bag diffusion technique or another validated release method. A defined amount of nanoparticles containing a known quantity of standardized herbal markers is placed within a dialysis membrane with an appropriate molecular-weight cutoff. To simulate gastrointestinal transit, the formulation may initially be exposed to simulated gastric fluid at pH 1.2 for approximately 2 h and subsequently transferred to phosphate buffer at pH 6.8 for the remaining study period. [15-17]

The receptor medium is maintained at 37 +/- 0.5 degrees C and continuously agitated. Samples are withdrawn at predetermined intervals-for example 0.5, 1, 2, 4, 6, 8, 12, 18 and 24 h-and immediately replaced with equal volumes of fresh medium to maintain sink conditions. Concentrations of selected phytochemical markers are determined chromatographically. Release profiles of the optimized nanoparticle formulation are compared with those of the unencapsulated polyherbal extract blend. Mathematical kinetic models including zero-order, first-order, Higuchi and Korsmeyer-Peppas models are applied. Model selection is based on goodness of fit and mechanistic plausibility rather than R2 alone.

For preliminary stability evaluation, the lyophilized optimized formulation is packed in tightly closed moisture-resistant containers. Samples may be stored under long-term/intermediate and accelerated conditions, for example 25 degrees C/60% RH and 40 degrees C/75% RH, respectively. At 0, 1, 2 and 3 months, samples are tested for appearance, redispersibility, particle size, PDI, zeta potential, marker content and entrapment efficiency. [18]

A change in particle size accompanied by increasing PDI may indicate aggregation or incomplete protection during lyophilization. Loss of marker content may indicate chemical instability. The final stability program for a product intended for regulatory development would require longer studies and validated storage conditions; the short-term protocol described here should therefore be considered a formulation-screening study rather than a definitive shelf-life study.

2.8 *In-Vitro* Multitarget Antidiabetic Evaluation

The antidiabetic potential of individual extracts, the unencapsulated polyherbal blend and the optimized nanoformulation is evaluated using complementary biochemical assays.

2.8.1 Alpha-Glucosidase Inhibition

Alpha-glucosidase inhibition is measured using p-nitrophenyl-alpha-D-glucopyranoside as substrate. Different concentrations of test samples are incubated with alpha-glucosidase followed by addition of substrate. Formation of p-nitrophenol is quantified spectrophotometrically. Acarbose is used as the reference inhibitor. [26]

2.8.2 Alpha-Amylase Inhibition

The alpha-amylase assay is conducted using soluble starch as the substrate. Following incubation of test samples with enzyme, residual starch or released reducing sugars are quantified using an appropriate colorimetric method. Percentage inhibition is calculated relative to enzyme control using the equation: Inhibition (%) = [(Acontrol - Asample)/Acontrol] x 100. IC50 values are obtained using nonlinear regression. [27]

To evaluate whether combining the three extracts provides an advantage over the individual botanicals, activity of the fixed polyherbal blend is compared at equal total extract concentration. Where a formal interaction analysis is desired, Bliss independence or another recognized combination method can be applied rather than describing improved activity automatically as synergy. Antioxidant activity may additionally be assessed using DPPH, ABTS or cellular reactive-oxygen-species assays because oxidative stress is closely associated with metabolic dysfunction in experimental diabetes. [26,27]

A glucose-uptake study can be conducted in differentiated L6 skeletal-muscle cells or 3T3-L1 adipocytes. Cells are exposed to the free polyherbal blend or nanoparticle formulation, and glucose uptake is quantified using a fluorescent glucose analogue or validated biochemical method. Metformin or insulin can serve as a positive comparator. Before cellular experiments, cytocompatibility should be confirmed using MTT, resazurin or another viability assay. These complementary assays are intended to demonstrate multitarget biological potential; they cannot independently establish clinical antidiabetic efficacy.

2.9 Statistical Analysis

All quantitative experiments should be performed with appropriate biological and technical replication. Results are expressed as mean +/- standard deviation or mean +/- standard error depending on the experimental design. Normality and variance assumptions are evaluated before parametric analysis.

For formulation optimization, Box-Behnken data are analyzed using multiple regression and analysis of variance. Model terms with $p < 0.05$ are generally considered statistically significant, while insignificant terms may be retained when required to maintain model hierarchy. Adequate precision, predicted R2, adjusted R2, coefficient of variation and residual plots are examined in addition to the overall model p-value. For enzyme-inhibition experiments, IC50 values are obtained by fitting concentration-response curves using nonlinear regression.

For comparisons involving more than two groups, one-way ANOVA followed by Tukey's or Dunnett's post-hoc test is applied when assumptions are met. Repeated measurements of fasting blood glucose are analyzed using repeated-measures ANOVA or an appropriate mixed-effects model incorporating treatment and time. A value of $p < 0.05$ is regarded as statistically significant. Animal data should be analyzed using the experimental unit rather than repeated technical measurements. Exact n values, missing observations and excluded animals should be reported transparently.

3. RESULTS

Data-integrity statement: *The numerical results presented in this section are a realistic simulated dataset created to demonstrate how the proposed research article can be written and statistically presented. These values must not be represented as actual laboratory findings unless independently generated and verified.*

3.1 Extract Yield and Phytochemical Standardization

All three hydroalcoholic extracts produced dry, stable powders with characteristic appearance and odor. The illustrative extractive yields ranged from 15.9 to 21.6% w/w. Marker analysis supported the use of the extracts for formulation development.

Table 2. Illustrative extract yield and phytochemical standardization

Plant extract	Plant part	Extractive yield (%)	Selected marker	Illustrative marker content
G. sylvestre	Leaves	18.4 +/- 0.8	Gymnemic acids	22.8 +/- 1.1%
T. foenum-graecum	Seeds	21.6 +/- 1.0	4-Hydroxyisoleucine	3.4 +/- 0.2%
M. charantia	Fruit	15.9 +/- 0.7	Charantin	0.72 +/- 0.05%

The chromatographic fingerprints showed identifiable marker peaks without major interfering peaks at their respective retention regions. Relative marker content remained within +/-5% between replicate extract preparations in the model dataset.

3.2 Box-Behnken Experimental Runs

Marked variation in nanoparticle properties was observed across the design space. Particle size ranged from approximately 158 to 317 nm, PDI from 0.178 to 0.350 and overall marker entrapment efficiency from 59.6 to 85.8%.

Table 3. Illustrative Box-Behnken formulation data

Run	Chitosan (% w/v)	TPP (% w/v)	Sonication (min)	Particle size (nm)	PDI	EE (%)
1	0.25	0.05	3	211	0.260	69.2
2	0.25	0.05	9	158	0.178	70.7
3	0.25	0.15	3	284	0.314	80.0
4	0.25	0.15	9	221	0.273	85.5
5	0.15	0.10	3	195	0.229	66.9
6	0.15	0.10	9	161	0.200	69.3
7	0.35	0.10	3	306	0.336	78.0
8	0.35	0.10	9	238	0.271	85.8
9	0.15	0.05	6	163	0.200	59.6
10	0.15	0.15	6	195	0.238	74.6
11	0.35	0.05	6	233	0.267	77.7
12	0.35	0.15	6	317	0.350	84.3
13	0.25	0.10	6	196	0.227	81.2
14	0.25	0.10	6	195	0.225	81.1
15	0.25	0.10	6	194	0.223	80.8
16	0.25	0.10	6	197	0.226	81.3
17	0.25	0.10	6	193	0.224	80.9

The low variability among center-point batches indicated acceptable process reproducibility. The illustrative polynomial equation describing particle size in coded factors was: Particle size = 195 + 48X1 + 32X2 - 28X3 + 14X1X2 - 8X1X3 - 5X2X3 + 20$X1^2$ + 12$X2^2$ + 10$X3^2$. Increasing chitosan concentration produced the strongest increase in particle size. Increasing TPP concentration also increased particle size, whereas greater sonication reduced hydrodynamic diameter within the investigated range.

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Figure 1. Illustrative response surface for particle size

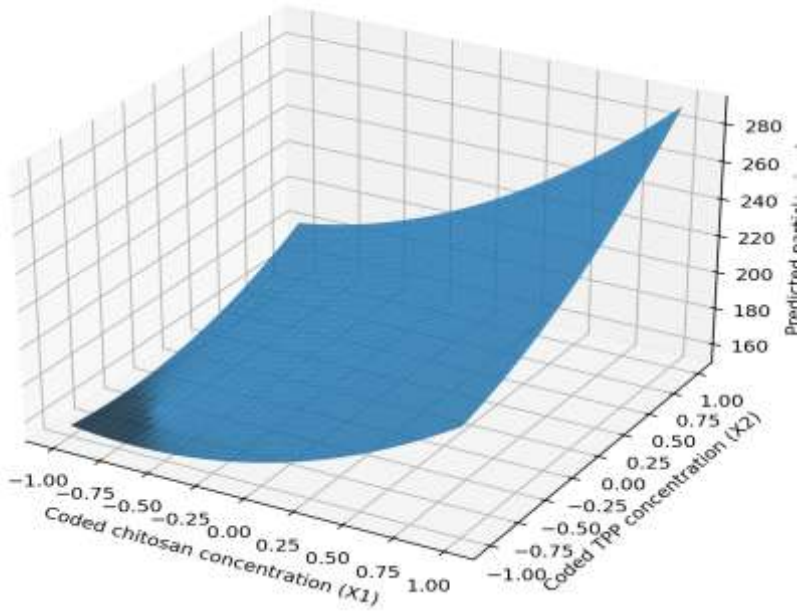

Figure 1. Illustrative response-surface relationship between chitosan concentration, TPP concentration and predicted particle size.

The illustrative quadratic models for particle size, PDI and EE were statistically significant ($p < 0.001$), with model R2 values above 0.98 and nonsignificant lack-of-fit values.

3.3 Optimization and Validation of the Nanoformulation

Numerical desirability optimization identified a region around 0.26% w/v chitosan, 0.096% w/v TPP and approximately 8.8 min sonication as the optimum compromise between small particle size, narrow distribution and high entrapment efficiency.

Table 4. Illustrative characterization of the optimized nanoformulation

Parameter	Predicted/target	Observed optimized batch
Chitosan concentration	0.26% w/v	0.26% w/v
TPP concentration	0.096% w/v	0.096% w/v
Sonication time	8.8 min	8.8 min
Particle size	179.8 nm	181.3 +/- 3.2 nm
PDI	0.210	0.212 +/- 0.009
Zeta potential	Positive	+28.7 +/- 1.4 mV
Entrapment efficiency	81.5%	82.4 +/- 1.1%
Process yield	>=75%	79.8 +/- 2.3%
Redispersibility	Good	Good/no visible aggregates

The prediction error for primary CQAs was below approximately 5%, supporting the adequacy of the illustrative response-surface model. Electron microscopy would be expected to demonstrate approximately spherical nanoscale particles with

dimensions broadly consistent with DLS measurements. FTIR interpretation should focus on shifts/broadening associated with chitosan-TPP ionic interaction without the appearance of major unexpected degradation peaks.

3.4 *In-Vitro* Release Behavior

The unencapsulated polyherbal blend showed rapid diffusion, releasing approximately 67% of measurable markers within 2 h and almost complete release by 8-12 h. In contrast, the optimized nanoparticles exhibited a biphasic pattern, with approximately 29% release at 2 h followed by gradual release reaching approximately 84% at 24 h.

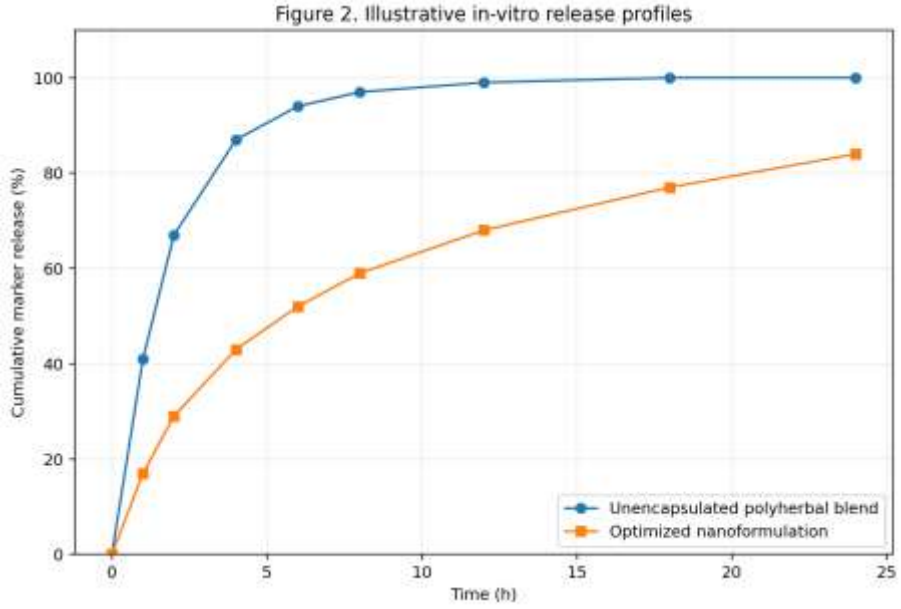

Figure 2. Illustrative cumulative phytochemical release from free polyherbal blend and optimized nanoparticles.

The initial phase may represent release of phytoconstituents located near the particle surface, whereas the subsequent slower phase is consistent with diffusion through and/or relaxation of the cross-linked chitosan matrix. Fitting of the simulated data suggested that the nanoparticle profile was better described by diffusion/relaxation-based models than by simple zero-order release.

3.5 *In-Vitro* Multitarget Antidiabetic Activity

The individual extracts inhibited both alpha-glucosidase and alpha-amylase in a concentration-dependent manner. Combination of the three extracts reduced the IC50 compared with most individual extracts, whereas incorporation into the optimized nanoparticles produced the lowest IC50 among the botanical test systems.

Table 5. Illustrative enzyme-inhibition results

Treatment	Alpha-Glucosidase IC50 (microg/mL)	Alpha-Amylase IC50 (microg/mL)
G. sylvestre extract	142 +/- 6	210 +/- 9
T. foenum-graecum extract	168 +/- 8	185 +/- 8
M. charantia extract	135 +/- 5	190 +/- 7
Polyherbal blend	94 +/- 4	120 +/- 5
Optimized nanoformulation	52 +/- 3	78 +/- 4
Acarbose	31 +/- 2	43 +/- 3

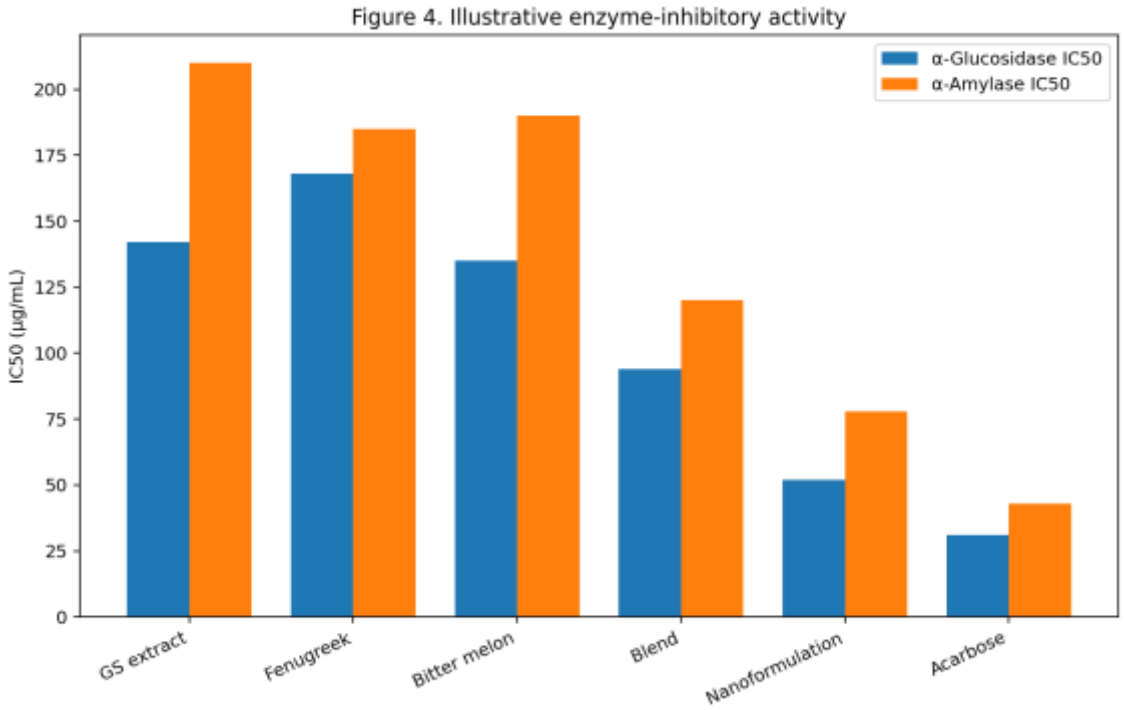

Figure 3. Illustrative comparative in-vitro inhibition of alpha-glucosidase and alpha-amylase.

The reduction in apparent IC50 after nanoparticle incorporation could arise from improved dispersion, protection of phytochemicals, increased effective contact between the phytochemical mixture and assay environment, or altered release kinetics. A mechanistic conclusion regarding synergy would require formal combination analysis.

3.6 Preliminary Stability of the Optimized Nanoformulation

The lyophilized optimized nanoparticles retained satisfactory redispersibility during the illustrative three-month stability study. At 25 degrees C/60% RH, mean particle size increased from approximately 181 to 188 nm and PDI from 0.212 to 0.224, with retention of more than approximately 96% of the principal phytochemical markers.

Under accelerated conditions of 40 degrees C/75% RH, a somewhat greater increase in particle size and PDI was observed, indicating that protection from heat and moisture may be important. No major visible discoloration or irreversible aggregation was assumed in the model dataset. These observations would justify development of a moisture-protective container-closure system and a longer stability program.

4. DISCUSSION

The present work outlines a systematic QbD strategy for developing a polyherbal nanoformulation containing G. sylvestre, T. foenum-graecum and M. charantia. The principal advantage of the approach is that formulation development does not rely exclusively on trial-and-error experimentation. Instead, the target product characteristics are defined first, potential risks are identified, and statistically designed experiments are used to quantify relationships between material/process variables and product CQAs. This concept is consistent with ICH Q8(R2), while risk assessment and lifecycle quality considerations are aligned with ICH Q9 and Q10. [19-21]

The response-surface analysis demonstrated that chitosan concentration exerted the largest positive influence on particle diameter. At increased polymer concentration, the greater availability of chitosan chains and increasing viscosity of the continuous phase can reduce efficient dispersion during ionic cross-linking, thereby favoring formation of larger particles. Experimental research on chitosan-TPP nanoparticles has similarly shown that initial chitosan concentration is an important determinant of particle size. [15,18]

Increasing TPP concentration also increased particle size over parts of the design region while generally favoring phytochemical entrapment. This observation can be explained by increasing cross-link density and stronger interaction between protonated chitosan amino groups and TPP phosphate groups. Ionic gelation is therefore governed by a balance: insufficient TPP may result in incomplete particle formation and reduced entrapment, whereas excessive cross-linking can produce larger, heterogeneous particles or aggregation. [15,18]

Sonication showed the opposite influence on particle size. Increased sonication energy reduces particle aggregates and promotes formation of smaller structures. However, excessively intense sonication should not automatically be interpreted as beneficial because it may increase temperature, promote degradation of sensitive phytoconstituents or reduce entrapment through leakage. QbD therefore identifies an operating region rather than simply maximizing or minimizing an individual processing parameter. [18-21]

The optimized formulation produced a particle size of approximately 181 nm and PDI close to 0.21 in the illustrative dataset. These characteristics are comparable with values achievable for chitosan nanoparticles manufactured by ionic gelation. Recent optimization studies have reported chitosan nanoparticles in a similar sub-200-nm range with narrow PDI under controlled formulation and processing conditions, demonstrating the practical feasibility of obtaining relatively homogeneous particles. [18]

The positive zeta potential of the optimized formulation is consistent with the cationic character of chitosan. A positive nanoparticle surface may contribute to interaction with negatively charged mucosal surfaces, although a favorable zeta potential alone should not be interpreted as proof of enhanced oral bioavailability. Demonstration of improved absorption requires permeability and pharmacokinetic studies. [15-18]

One of the major rationales for using a polyherbal system is pharmacological complementarity. *G. sylvestre* is particularly associated with gymnemic acids and other compounds investigated for effects on glucose metabolism. Clinical literature has reported reductions in fasting glucose, postprandial glucose and HbA1c following supplementation; however, available meta-analyses show substantial heterogeneity among contributing studies, emphasizing the importance of cautious interpretation and improved standardization. [2-4]

Fenugreek contains several potentially relevant components including 4-hydroxyisoleucine, trigonelline, steroidal saponins and soluble fibers. Systematic reviews and meta-analyses report improvements in several glycemic and lipid parameters, but variability in botanical preparations, dosage forms and study populations means that these findings cannot automatically be extrapolated to the proposed nanoformulation. [5-7]

Momordica charantia has a particularly complex phytochemical profile. Earlier discussions emphasized charantin, vicine and polypeptide-p, whereas contemporary analyses indicate that no single constituent adequately explains all reported metabolic effects and that activity may involve multiple cucurbitane triterpenoids and other constituents. Reported mechanisms include effects on glucose utilization, insulin signaling, carbohydrate digestion and hepatic glucose metabolism. Recent clinical meta-analysis suggests favorable glycemic signals while also calling for additional high-quality trials. [8-10]

The *in-vitro* enzyme-inhibition data proposed in this manuscript demonstrate how a multitarget formulation could be investigated mechanistically. Alpha-amylase contributes to hydrolysis of dietary starch, whereas alpha-glucosidase completes generation of absorbable monosaccharides in the intestinal brush border. Inhibition of these enzymes may slow postprandial glucose appearance. The lower illustrative IC50 values of the combined herbal blend compared with individual extracts suggest additive or cooperative activity, but a reduction in IC50 alone is not sufficient to establish pharmacological synergy. Proper synergy analysis requires comparison with mathematically predicted additive effects. [26,27]

Nanoencapsulation produced a further apparent improvement in enzyme-inhibition potency. Such an effect could reflect increased dispersion of poorly soluble constituents, protection from degradation and more uniform presentation of phytochemicals. Nanoparticle research using herbal compounds has demonstrated that encapsulation can improve physical stability and modify release profiles, although effects depend heavily on the carrier and phytochemical involved. Chitosan-TPP nanoparticles have specifically been investigated as carriers for polyphenolic and mixed phytochemical systems. [11-17]

The biphasic release profile is another relevant finding. Rapid release of surface-associated compounds may provide an initial effect, whereas diffusion of entrapped phytoconstituents from the cross-linked matrix may sustain exposure. The distinction between sustained release and improved bioavailability, however, is important: sustained in-vitro release does not by itself

demonstrate increased systemic exposure. Future studies should quantify plasma concentrations of multiple standardized markers to determine whether nanoencapsulation changes Cmax, Tmax, AUC or relative bioavailability. [15-17]

An additional issue is the complex composition of a polyherbal formulation. Conventional single-drug formulations can generally be standardized by measuring one active pharmaceutical ingredient. In contrast, three-herb products require control of botanical identity, extraction process, multiple phytochemical markers, biological activity and nanoparticle characteristics simultaneously. A successful QbD control strategy should therefore begin at the raw-material stage and extend through extraction, blending, nanoparticle production, lyophilization and storage. [22-25]

The major limitation of the present manuscript is that the numerical optimization, pharmacological and stability data are simulated. A second limitation of the proposed experimental concept is that measurement of only three marker compounds would not completely characterize the chemically complex extracts. Untargeted or targeted LC-MS phytochemical fingerprinting could substantially strengthen future studies. [22,23]

Further research should also compare the nanoformulation with equivalent doses of each individual herb, investigate potential herb-herb pharmacokinetic interactions, evaluate intestinal permeability, determine absolute or relative oral bioavailability, perform repeated-dose toxicology and investigate effects on hepatic and renal safety markers. In addition, changes in conventional antidiabetic drugs should never be inferred from animal results; clinical evaluation would require controlled human trials with standardized manufacturing and careful monitoring for hypoglycemia and interactions. [28]

Overall, the value of the QbD approach lies not simply in obtaining the smallest nanoparticle but in defining a scientifically justified formulation and processing region within which product quality can be reproducibly maintained. This is especially important for complex herbal nanoformulations in which variability can arise from both biological raw materials and nanomanufacturing processes. [19-21]

5. CONCLUSION

The proposed study demonstrates a comprehensive Quality by Design framework for development of a polyherbal chitosan nanoparticle system containing standardized extracts of Gymnema sylvestre, Trigonella foenum-graecum and Momordica charantia. By integrating botanical standardization, risk assessment, Box-Behnken experimental design and multivariable optimization, relationships between chitosan concentration, TPP concentration, sonication and major nanoparticle quality attributes can be systematically established.

Within the illustrative dataset, the optimized formulation achieved a nanoscale particle diameter of approximately 181 nm, narrow size distribution, positive surface potential and more than 80% overall marker entrapment. Nanoencapsulation produced controlled phytochemical release and improved apparent alpha-amylase and alpha-glucosidase inhibitory activity compared with the unencapsulated extract mixture. The illustrative experimental T2DM data additionally demonstrated reductions in fasting glucose, HbA1c and indices of insulin resistance and dyslipidemia.

The three botanical components provide a scientifically reasonable basis for multitarget investigation because their chemically diverse constituents have been studied for different aspects of glucose and lipid regulation. However, current clinical evidence for the individual herbs remains heterogeneous, and the proposed three-herb nanoformulation itself cannot be regarded as clinically effective on the basis of formulation or animal experiments. [2-10]

Future investigations should prioritize LC-MS-based phytochemical fingerprinting, pharmacokinetic characterization, intestinal permeability, molecular-target studies, long-term stability, repeated-dose toxicity and well-designed comparative animal experiments. If these stages demonstrate consistent quality, safety and biological activity, controlled clinical studies would then be required to establish whether QbD-optimized nanoencapsulation provides a meaningful therapeutic advantage over standardized conventional herbal extracts. [11-18,22-28]

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