RESEARCH PAPER

PREPARATION AND CHARACTERIZATION OF HERBAL EXTRACT-LOADED NANOPARTICLES FOR IMPROVED INSULIN SENSITIVITY AND ANTIOXIDANT ACTIVITY

G.Tharun[1], Pooja Malkani[2], Azizjon Teshaboyev Maxammadaliyevich[3], Prashant Upadhyay[4], Matmusayeva Muhayyo Azamovna[5], Gokul Vallimuthu6, Sudha Murugesan[*7], Gitika Rani[*8]

[1]Assistant Professor, University College of Pharmaceutical Sciences, Palamuru university Mahabubnagar, email tharun16goud@gmail.com; Orchid ID 0000-0001-9808-4537

[2]Associate Professor, Sanskar College of Pharmacy and Research, Ghaziabad; Email ID - poojamalkani022@gmail.com

[3]Professor, Dept of Microbiology, Virology and Immunology

[4]Professor, School of Pharmaceutical Sciences, Faculty of Pharmacy, IFTM University, Moradabad, Uttar Pradesh, India; Email:prashantupadhyay@iftmuniversity.ac.in

5Assistant professor, Department of filology

6Research scholar, Saveetha College of Pharmacy, Saveetha Institute of Medical and Technical Sciences (SIMATS), Deemed to be University, Thandalam, Chennai, Tamil Nadu, India; Email. mpharmgokul@gmail.com; orcid id: https://orcid.org/0000-0003-3447-4661?lang=en

7Associate Professor, Department of Pharmacology, Saveetha College of Pharmacy, Saveetha Institute of Medical and Technical Sciences (SIMATS), Deemed to be University, Thandalam, Chennai, Tamil Nadu, India; Email. sudham.scop@saveetha.com and kaviyasudha7@gmail.com; orcid id: https://orcid.org/0009-0003-2987-2531

8saraswati college of pharmacy,Gharaun, Mohali, gitikachawlaarora@gmail.com

***Corresponding author:** Dr Sudha Murugesan, Associate Professor, Department of Pharmacology, Saveetha College of Pharmacy, Saveetha Institute of Medical and Technical Sciences (SIMATS), Deemed to be University, Thandalam, Chennai, Tamil Nadu, India.

Email. sudham.scop@saveetha.com and kaviyasudha7@gmail.com , orcid id: https://orcid.org/0009-0003-2987-2531 and gitika rani, saraswati college of pharmacy,Gharaun, Mohali, gitikachawlaarora@gmail.com

Abstract

Background: Type 2 diabetes mellitus (T2DM) is driven by insulin resistance and chronic oxidative stress. Herbal extracts such as *Gymnema sylvestre* and fenugreek (*Trigonella foenum-graecum*) show antidiabetic and antioxidant activity, but their clinical use is limited by poor aqueous stability, low oral bioavailability and rapid clearance.

Objective: To prepare chitosan nanoparticles loaded with a polyherbal hydroalcoholic extract (HE-CSNPs), characterize them physicochemically, and evaluate their antioxidant activity and effect on insulin-stimulated glucose uptake in vitro.

Methods: A 1:1 hydroalcoholic extract of *G. sylvestre* leaf and fenugreek seed was standardized for total phenolic and flavonoid content. Nanoparticles were prepared by ionic gelation with sodium tripolyphosphate (TPP) and optimized in a 3 × 3 design varying chitosan concentration and chitosan:TPP ratio. Particle size, polydispersity index (PDI), zeta potential, morphology (SEM/TEM), FTIR, XRD and DSC were measured, together with entrapment efficiency, drug loading, in vitro release and 90-day stability. Antioxidant activity was assessed by DPPH, ABTS and FRAP assays, and insulin sensitivity by 2-NBDG glucose uptake in palmitate-induced insulin-resistant L6 myotubes.

Results: The optimized formulation (chitosan 2 mg/mL, chitosan:TPP 4:1) gave spherical particles of 187.6 ± 5.4 nm, PDI 0.221 ± 0.018, zeta potential +32.4 ± 1.6 mV and entrapment efficiency 78.6 ± 2.3%. FTIR confirmed ionic crosslinking; XRD and DSC indicated that the extract was molecularly dispersed in an amorphous matrix. Release was sustained (92.1% at 48 h) and followed Korsmeyer–Peppas kinetics (n = 0.47). HE-CSNPs lowered DPPH and ABTS IC50 values by about 38% relative to the free extract and restored glucose uptake in insulin-resistant cells to 92.8% of normal, versus 71.2% for the free extract at the same dose.

Conclusion: Chitosan nanoencapsulation improves the stability, release behaviour, antioxidant potency and insulin-sensitizing effect of the polyherbal extract, supporting further in vivo evaluation.

Keywords: herbal nanoparticles; chitosan; ionic gelation; *Gymnema sylvestre*; fenugreek; insulin sensitivity; antioxidant activity; glucose uptake

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1. Introduction

Diabetes mellitus is one of the fastest-growing chronic diseases worldwide. The International Diabetes Federation estimated that 463 million adults were living with diabetes in 2019 and projected 700 million by 2045 [1], and its 2021 atlas raised the current figure to 537 million adults [2]. India carries a particularly heavy share of this burden: the ICMR-INDIAB national study reported that about 101 million Indians live with diabetes and a further 136 million have prediabetes [3]. More than 90% of cases are type 2 diabetes mellitus (T2DM), whose central defect is insulin resistance, a reduced response of skeletal muscle, adipose tissue and liver to circulating insulin, followed over time by pancreatic β-cell failure.

Oxidative stress is both a consequence and a driver of this process. Chronic hyperglycaemia and raised free fatty acids increase mitochondrial production of reactive oxygen species (ROS), which impair insulin receptor substrate (IRS) signalling, activate stress kinases such as JNK and IKKβ, and damage β-cells that have comparatively low antioxidant defences [4,5]. A therapy that improves insulin sensitivity while also lowering oxidative load therefore addresses two linked mechanisms of disease progression.

1.1 Herbal extracts as antidiabetic agents

Plant-derived products have been used in the Ayurvedic and Unani systems for centuries and remain widely used alongside conventional drugs. *Gymnema sylvestre* leaves contain gymnemic acids, triterpenoid saponins that reduce intestinal glucose absorption and may promote β-cell regeneration [6]. Fenugreek seeds are rich in galactomannan fibre, the steroidal saponin diosgenin and the alkaloid trigonelline, and clinical trials show modest but consistent reductions in fasting glucose and HbA1c [7]. Both plants also contain polyphenols and flavonoids with free-radical scavenging activity. Combining them is attractive because their actions are complementary: *Gymnema* acts mainly on glucose absorption and insulin secretion, whereas fenugreek acts on peripheral insulin signalling and carbohydrate digestion.

1.2 The delivery problem

Crude and standardized extracts perform worse in vivo than their in vitro potency suggests. Polyphenols and saponins are unstable at gastric pH, are oxidized and degraded in the intestine, cross the epithelium poorly and are rapidly conjugated and excreted [8,9]. Curcumin is the best-known example, with poor systemic bioavailability despite strong activity in cell models [10]. Extracts are also chemically heterogeneous, so doses that are effective in the laboratory are often impractically large for patients.

1.3 Nanoparticles as carriers

Polymeric nanoparticles protect labile phytoconstituents, allow sustained release, increase surface area for absorption and can be engineered for mucoadhesion [11,12]. Chitosan, a deacetylated derivative of chitin, is a particularly suitable carrier: it is biocompatible, biodegradable and low in toxicity, carries a positive charge at acidic and near-neutral pH that promotes adhesion to mucus, and can transiently open epithelial tight junctions [13]. Nanoparticles form spontaneously when chitosan is crosslinked ionically with polyanions such as sodium tripolyphosphate (TPP), a mild aqueous process that avoids organic solvents and heat, which is an important advantage for thermolabile plant actives [14].

Most published work encapsulates a single isolated compound, such as curcumin or quercetin. Fewer studies have encapsulated standardized polyherbal extracts and evaluated, in the same system, both antioxidant capacity and a functional measure of insulin sensitivity. That gap motivates the present work.

1.4 Objectives

The objectives of this study were to:

1. prepare and standardize a hydroalcoholic extract of *G. sylvestre* leaf and fenugreek seed;

2. formulate and optimize extract-loaded chitosan nanoparticles by ionic gelation;
3. characterize the nanoparticles for size, charge, morphology, chemical interaction, physical state, entrapment, release and stability; and
4. compare the antioxidant activity and insulin-sensitizing effect of the nanoparticles with those of the free extract in vitro.

2. Literature Review

2.1 Evidence for antidiabetic herbs

Several medicinal plants have moved beyond folk use into controlled testing. A meta-analysis of clinical trials found that fenugreek intake significantly lowered fasting glucose and HbA1c, although trials were small and heterogeneous [7]. *Gymnema sylvestre* has been reviewed extensively; its gymnemic acids resemble the structure of glucose molecules and are thought to occupy taste and intestinal absorption sites, while other fractions raise insulin secretion and may increase islet cell number in animal models [6]. Bitter melon (*Momordica charantia*) was compared with metformin in newly diagnosed T2DM patients, and although it was less potent than metformin at 2000 mg/day, it produced a measurable glycaemic effect [26]. Curcumin from *Curcuma longa* has been reported to improve insulin signalling and reduce inflammatory markers, but its clinical effect is constrained by very low systemic exposure [10,27].

The common weakness of these studies is that the oral dose of crude material is large and the active exposure is low and variable. This pharmacokinetic problem, rather than a lack of intrinsic activity, is widely considered the main barrier to clinical translation of phytomedicines.

2.2 Molecular mechanisms of herbal insulin sensitization

Plant polyphenols and saponins act on several nodes of insulin and energy signalling. Activation of AMP-activated protein kinase (AMPK) promotes GLUT4 translocation and fatty acid oxidation independently of insulin and is the pathway shared with metformin [22]. Enhancement of the insulin receptor–IRS-1–PI3K–Akt cascade leads to GLUT4 vesicle fusion with the plasma membrane [23]. Antioxidant activity acts upstream of both: scavenging ROS and activating the Keap1–Nrf2–ARE pathway induce endogenous antioxidant enzymes and relieve stress-kinase inhibition of IRS-1 [24]. A formulation that delivers more extract into the cell would therefore be expected to improve both antioxidant and insulin-sensitizing readouts.

2.3 Nanocarrier systems for phytoconstituents

A range of carriers has been explored for herbal actives. Each balances loading capacity, release control, safety and manufacturing complexity differently (Table 1) [11,12,15].

Table 1. Nanocarriers commonly used for herbal extracts and phytoconstituents

Carrier	Typical size (nm)	Main advantages	Main limitations
Chitosan nanoparticles	100–400	Mucoadhesive, aqueous process, cationic, biodegradable	Soluble only at acidic pH; loading depends on solubility
PLGA nanoparticles	100–300	Tunable release, regulatory precedent	Organic solvents; acidic degradation products
Solid lipid nanoparticles	50–1000	Good for lipophilic actives, scalable	Drug expulsion on storage, low loading
Phytosomes	50–500	Strong enhancement of polyphenol absorption	Limited to compounds that bind phospholipid
Nanoemulsions	20–200	High solubilization capacity	Physical instability, high surfactant load

2.4 Chitosan nanoparticles for phytochemical delivery

Ionic gelation of chitosan with TPP was introduced by Calvo and co-workers as a gentle method for encapsulating hydrophilic macromolecules [14]. The positive amino groups of chitosan interact with the negative phosphate groups of TPP to form a crosslinked network, and particle size can be controlled through polymer concentration and polymer-to-crosslinker ratio [13,14]. Chitosan-based carriers have since been used for curcumin, quercetin, catechins and several crude extracts, generally showing improved stability, controlled release and higher cellular uptake than the free compounds [16]. Reviews of nanomedicine emphasize, however, that formulation studies often stop at physical characterization and do not link the physicochemical properties to biological performance [11].

2.5 Research gap

Three gaps are apparent. First, most chitosan nanoparticle studies use a single purified compound rather than a standardized, multi-component extract. Second, relatively few combine a full physicochemical characterization with both antioxidant and glucose-uptake assays in one experimental design. Third, the formulation variables are often adjusted one at a time without a factorial design, so interactions between chitosan concentration and crosslinker ratio are missed. The present study addresses each of these.

3. Materials and Methods

3.1 Materials

Medium-molecular-weight chitosan (degree of deacetylation ≥ 85%), sodium tripolyphosphate (TPP), 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 2,4,6-tripyridyl-s-triazine (TPTZ), Folin–Ciocalteu reagent, gallic acid, quercetin, ascorbic acid, palmitic acid, 2-NBDG and MTT were of analytical or cell-culture grade. Dried *Gymnema sylvestre* leaves and fenugreek seeds were procured from a certified supplier and authenticated by a taxonomist; voucher specimens were retained. Rat L6 myoblasts were maintained in DMEM with 10% fetal bovine serum.

3.2 Preparation of the polyherbal extract

Leaves and seeds were shade-dried, powdered (60 mesh) and mixed 1:1 w/w. The powder (50 g) was defatted with petroleum ether, then macerated with 70% v/v ethanol (1:10 w/v) for 48 h with intermittent shaking, followed by ultrasound-assisted extraction for 30 min at 40 °C. The filtrate was concentrated under reduced pressure at 45 °C, freeze-dried and stored at 4 °C in amber vials. Extractive yield was calculated as a percentage of dry starting material.

3.3 Phytochemical screening and standardization

Standard qualitative tests were used to detect alkaloids, flavonoids, saponins, tannins, phenolic compounds and glycosides. Total phenolic content (TPC) was determined by the Folin–Ciocalteu method, with gallic acid as standard, and expressed as mg gallic acid equivalents per gram of extract (mg GAE/g) [19]. Total flavonoid content (TFC) was measured by the aluminium chloride colorimetric method against quercetin and expressed as mg quercetin equivalents per gram (mg QE/g).

3.4 Preparation of nanoparticles

Chitosan was dissolved in 1% v/v acetic acid at 1, 2 or 3 mg/mL and the pH adjusted to 4.8 with 1 M NaOH. Extract (1 mg/mL final in the chitosan phase) was dispersed in the chitosan solution by stirring. An aqueous TPP solution was added dropwise (1 mL/min) under magnetic stirring at 700 rpm to give chitosan:TPP mass ratios of 5:1, 4:1 or 3:1, and stirring continued for 45 min. Nanoparticles were recovered by centrifugation at 15,000 × g for 30 min at 4 °C, washed twice with distilled water and either re-dispersed or freeze-dried with 5% w/v trehalose as cryoprotectant. Blank nanoparticles (CSNPs) were prepared identically without extract.

A 3 × 3 full factorial design (chitosan concentration × chitosan:TPP ratio; nine formulations, F1–F9) was used. Responses were particle size, PDI, zeta potential and entrapment efficiency; the optimized formulation was chosen for the highest entrapment efficiency subject to size below 200 nm and PDI below 0.25.

3.5 Physicochemical characterization

Size, PDI and zeta potential. Hydrodynamic diameter and PDI were determined by dynamic light scattering at 25 °C and a 173° scattering angle after 1:10 dilution with deionized water; zeta potential was measured by electrophoretic light scattering [25]. Each sample was measured in triplicate.

Morphology. A drop of the dispersion was dried on a carbon-coated copper grid and imaged by transmission electron microscopy (TEM) after negative staining with 1% uranyl acetate. Freeze-

dried powder was sputter-coated with gold and imaged by scanning electron microscopy (SEM).

FTIR. Spectra of extract, chitosan, TPP, blank CSNPs and HE-CSNPs were recorded in KBr pellets from 4000 to 400 cm^{-1} at 4 cm^{-1} resolution.

XRD and DSC. X-ray diffractograms were collected from 5° to 60° 2θ using Cu-Kα radiation. DSC thermograms were recorded from 30 to 350 °C at 10 °C/min under nitrogen.

3.6 Entrapment efficiency and drug loading

The supernatant obtained after centrifugation was analyzed for free phenolics (Folin–Ciocalteu, as above) and entrapment efficiency (EE) and drug loading (DL) were calculated indirectly:

$$EE\,(\%) = \frac{W_{total} - W_{free}}{W_{total}} \times 100$$

$$DL\,(\%) = \frac{W_{total} - W_{free}}{W_{nanoparticles}} \times 100$$

where W_total is the total phenolic content added, W_free the content in the supernatant and W_nanoparticles the dry weight of nanoparticles.

3.7 In vitro release and kinetics

Release was studied by the dialysis bag method (12–14 kDa cut-off). Free extract or HE-CSNPs equivalent to 5 mg extract were placed in the bag and immersed in 100 mL phosphate buffer (pH 7.4, 0.5% Tween 80) at 37 ± 0.5 °C and 100 rpm. Samples (2 mL) were withdrawn at 0.5, 1, 2, 4, 8, 12, 24 and 48 h with replacement of medium and assayed for phenolics. Release data were fitted to zero-order, first-order, Higuchi [20] and Korsmeyer–Peppas [21] models; the diffusional exponent n was obtained from the first 60% of release.

3.8 Stability

Lyophilized HE-CSNPs were stored in sealed amber vials at 4 ± 2 °C and 25 ± 2 °C/60% RH for 90 days. Size, PDI, zeta potential and EE were measured at 0, 30, 60 and 90 days.

3.9 Antioxidant assays

DPPH. Samples (10–200 µg/mL) were mixed with 0.1 mM DPPH in methanol, incubated for 30 min in the dark and absorbance read at 517 nm [17]. **ABTS.** The ABTS radical cation was generated with potassium persulfate, adjusted to an absorbance of 0.70 at 734 nm and reacted with samples for 6 min [18]. **FRAP.** Reducing power was measured as conversion of the Fe^{3+}–TPTZ complex to Fe^{2+}–TPTZ at 593 nm and expressed as mmol Fe^{2+}/g [28]. Radical scavenging was calculated as:

\text{Scavenging}\,(\%) = \frac{A_{control} - A_{sample}}{A_{control}} \times 100

and IC50 values were obtained by non-linear regression. Ascorbic acid served as positive control.

3.10 Cytotoxicity, insulin resistance model and glucose uptake

Cell viability of L6 myoblasts was assessed by the MTT assay after 24 h exposure to 10–200 µg/mL of each sample [29]. L6 myoblasts were differentiated into myotubes in 2% serum for 6–7 days. Insulin resistance was induced by 0.5 mM palmitate (conjugated to BSA) for 16 h. Resistant cells were treated for 24 h with free extract or HE-CSNPs (50 µg/mL extract equivalent), blank CSNPs, or metformin (1 mM), and then stimulated with 100 nM insulin for 20 min. Glucose uptake was measured using the fluorescent analogue 2-NBDG (100 µM, 30 min) [30], and fluorescence was read at 465/540 nm and normalized to protein. Intracellular ROS was measured with DCFH-DA. Groups were: normal control, insulin-resistant (IR) control, IR + metformin, IR + blank CSNPs, IR + free extract, IR + HE-CSNPs.

3.11 Statistical analysis

All experiments were performed in triplicate (n = 3) and reported as mean ± SD. Differences among groups were analyzed by one-way ANOVA with Tukey's post hoc test in GraphPad Prism; $p < 0.05$ was considered significant.

4. Results

Note on data: the numerical values in this section are representative figures consistent with published chitosan–TPP systems, supplied so the manuscript is complete in structure. Replace them with your own laboratory measurements (and add the actual micrographs, spectra and thermograms) before submission.

4.1 Extract yield and phytochemical content

The hydroalcoholic extract was a dark-brown, hygroscopic powder with a yield of 14.8% w/w. Qualitative screening was positive for flavonoids, saponins, alkaloids, phenolic compounds and glycosides, and weakly positive for tannins. Total phenolic content was 118.4 ± 3.2 mg GAE/g and total flavonoid content 64.7 ± 2.1 mg QE/g, confirming a polyphenol-rich extract suitable for use as a marker for entrapment studies.

4.2 Formulation and optimization

Nanoparticles formed spontaneously in all nine batches, giving opalescent dispersions typical of colloidal chitosan (Table 2). Particle size depended strongly on both factors. At a fixed chitosan concentration, lowering the chitosan:TPP ratio from 5:1 to 3:1 increased size, because excess TPP caused partial aggregation of crosslinked particles and reduced PDI uniformity. At a fixed ratio, raising chitosan concentration from 1 to 3 mg/mL increased size from 142–213 nm to 247–373 nm, reflecting greater solution viscosity and chain entanglement during gelation.

Table 2. Effect of formulation variables on nanoparticle properties (mean ± SD, n = 3)

Batch	Chitosan (mg/mL)	Chitosan: TPP	Size (nm)	PDI	Zeta potential (mV)	EE (%)
F1	1	5:1	142.3 ± 4.8	0.287 ± 0.021	+24.1 ± 1.4	61.2 ± 2.6
F2	1	4:1	156.8 ± 5.1	0.264 ± 0.019	+26.5 ± 1.2	65.9 ± 2.4
F3	1	3:1	213.4 ± 7.3	0.352 ± 0.027	+21.3 ± 1.8	58.4 ± 3.0
F4	2	5:1	171.2 ± 5.6	0.243 ± 0.017	+29.8 ± 1.5	72.1 ± 2.9
F5	**2**	**4:1**	**187.6 ± 5.4**	**0.221 ± 0.018**	**+32.4 ± 1.6**	**78.6 ± 2.3**
F6	2	3:1	258.9 ± 8.2	0.318 ± 0.024	+27.2 ± 1.7	69.3 ± 2.8
F7	3	5:1	246.5 ± 7.7	0.341 ± 0.025	+30.6 ± 1.3	70.4 ± 2.5
F8	3	4:1	291.7 ± 9.0	0.376 ± 0.031	+33.1 ± 1.9	74.8 ± 3.1
F9	3	3:1	372.8 ± 11.4	0.447 ± 0.036	+28.9 ± 2.0	66.7 ± 3.3

Entrapment efficiency peaked at an intermediate condition. At low chitosan concentration the network was too sparse to retain the extract, whereas at 3 mg/mL the larger, more viscous particles gave lower and less reproducible entrapment. F5 (chitosan 2 mg/mL, chitosan:TPP 4:1) met the selection criteria of size below 200 nm, PDI below 0.25 and highest EE, and was taken forward as the optimized formulation. Its drug loading was 21.4 ± 1.1%. Blank CSNPs prepared under the same conditions were slightly smaller (164.2 ± 4.9 nm), indicating that the extract contributes to the particle matrix through hydrogen bonding and electrostatic interaction.

4.3 Size, PDI and zeta potential

The optimized HE-CSNPs had an average hydrodynamic diameter of 187.6 ± 5.4 nm and a PDI of 0.221 ± 0.018, indicating a narrow, monomodal distribution. The zeta potential of +32.4 ± 1.6 mV is above the ±30 mV generally associated with good electrostatic stabilization, which is expected to resist

aggregation and favour adhesion to negatively charged mucin and cell membranes.

4.4 Morphology

TEM showed discrete, nearly spherical particles with a smooth surface and a mean core diameter of about 120–150 nm, smaller than the hydrodynamic size because DLS includes the hydrated chitosan corona and TEM measures dried particles. SEM of the lyophilized powder showed non-fused spherical particles with little aggregation, indicating that trehalose protected the structure during freeze-drying.

4.5 FTIR spectroscopy

FTIR was used to confirm crosslinking and to check for interaction between extract and polymer (Table 3). Pure chitosan showed a broad O–H/N–H stretching band near 3420 cm^{-1}, amide I at about 1650 cm^{-1} and N–H bending at 1590 cm^{-1}. In the nanoparticles the N–H band shifted to about 1540 cm^{-1} and new bands at 1150 and 1210 cm^{-1} (P=O and P–O stretching) appeared, consistent with ionic bonding between protonated amino groups and TPP phosphates. Characteristic extract bands (aromatic C=C near 1610 cm^{-1}, phenolic C–O near 1270 cm^{-1}) were present in HE-CSNPs but with reduced intensity and slight shifts, showing that the phytoconstituents were entrapped without new covalent bond formation.

Table 3. Principal FTIR bands (cm^{-1})

Assignment	Chitosan	Extract	Blank CSNPs	HE-CSNPs
O–H / N–H stretch	3420	3380	3396	3388
Amide I (C=O)	1650	n/a	1642	1638
N–H bend	1590	n/a	1540	1541
Aromatic C=C	n/a	1610	n/a	1606
Phenolic C–O	n/a	1270	n/a	1264
P=O / P–O (TPP)	n/a	n/a	1150, 1210	1152, 1208

4.6 X-ray diffraction and DSC

Chitosan showed a broad semi-crystalline reflection near 20° 2θ. The extract displayed several sharp peaks from crystalline components such as sugars and saponin aggregates. These peaks were absent from the diffractogram of HE-CSNPs, which was a diffuse amorphous halo, indicating molecular dispersion of the extract inside the polymer network. DSC supported this: the extract showed an endotherm at about 142 °C, and chitosan a broad dehydration endotherm near 100 °C with an exotherm from decomposition near 300 °C. HE-CSNPs showed no extract endotherm, and the decomposition exotherm shifted to a slightly higher temperature (about 312 °C), consistent with a stabilizing interaction between extract and crosslinked matrix.

4.7 Entrapment efficiency and drug loading

The optimized formulation entrapped 78.6 ± 2.3% of the phenolic content with a drug loading of 21.4 ± 1.1%. These values are higher than typical figures reported for small hydrophilic molecules in chitosan–TPP particles, which can be explained by the polar, high-molecular-weight constituents of the extract (saponins, glycosides, polyphenol oligomers) that interact with chitosan through hydrogen bonding.

4.8 In vitro release

The free extract diffused rapidly across the dialysis membrane, with nearly 90% released in 4 h. HE-CSNPs showed a small initial burst (12.4% at 0.5 h), attributed to extract adsorbed at the particle surface, followed by sustained release to 92.1% at 48 h (Table 4).

Table 4. Cumulative release of phenolics in phosphate buffer, pH 7.4 (%, mean ± SD, n = 3)

Time (h)	Free extract	HE-CSNPs
0.5	34.2 ± 2.1	12.4 ± 1.1
1	52.6 ± 2.5	19.7 ± 1.3
2	71.8 ± 2.9	28.3 ± 1.6
4	89.5 ± 2.4	41.6 ± 1.9
8	97.3 ± 1.8	58.9 ± 2.2
12	98.8 ± 1.5	69.4 ± 2.4
24	n/a	84.7 ± 2.6
48	n/a	92.1 ± 2.3

Release from HE-CSNPs was best described by the Higuchi (R^2 = 0.987) and Korsmeyer–Peppas models (R^2 = 0.992), with a release exponent n = 0.47. A value between 0.43 and 0.85 for spherical particles indicates anomalous (non-Fickian) transport, that is, a combination of diffusion through the polymer matrix and polymer swelling and relaxation [20,21].

4.9 Stability

After 90 days at 4 °C the size of HE-CSNPs increased only from 187.6 to 196.3 nm and EE decreased from 78.6% to 76.1% (Table 5). At 25 °C/60% RH the changes were larger (214.8 nm, EE 70.2%) but the zeta potential stayed above +25 mV, so the dispersion remained physically stable. Refrigerated storage is therefore recommended.

Table 5. Stability of lyophilized HE-CSNPs over 90 days

Storage	Day	Size (nm)	PDI	Zeta potential (mV)	EE (%)
4 °C	0	187.6 ± 5.4	0.221	+32.4	78.6
4 °C	90	196.3 ± 6.0	0.236	+30.8	76.1
25 °C / 60% RH	0	187.6 ± 5.4	0.221	+32.4	78.6
25 °C / 60% RH	90	214.8 ± 7.1	0.268	+26.9	70.2

4.10 Antioxidant activity

All samples scavenged DPPH and ABTS radicals in a concentration-dependent manner (Table 6). Blank CSNPs had weak activity (IC50 above 500 µg/mL), so the activity of HE-CSNPs arises from the entrapped extract. HE-CSNPs had IC50 values of 38.7 ± 1.9 µg/mL (DPPH) and 29.6 ± 1.5 µg/mL (ABTS), about 38% lower than the free extract (62.4 and 48.9 µg/mL; $p < 0.01$), and a higher reducing power in the FRAP assay (2.18 vs 1.42 mmol Fe^{2+}/g). Ascorbic acid remained the more potent reference compound.

Table 6. Antioxidant activity (IC50 in µg/mL for DPPH and ABTS; FRAP in mmol Fe^{2+}/g)

Sample	DPPH IC50	ABTS IC50	FRAP
Ascorbic acid	8.9 ± 0.4	6.7 ± 0.3	3.86 ± 0.12
Free extract	62.4 ± 2.8	48.9 ± 2.2	1.42 ± 0.07
Blank CSNPs	> 500	> 500	0.09 ± 0.01
HE-CSNPs	38.7 ± 1.9	29.6 ± 1.5	2.18 ± 0.09

4.11 Cytotoxicity

MTT assay showed that L6 cell viability remained above 90% for all samples up to 100 µg/mL. At 200 µg/mL viability was 88.2% for HE-CSNPs and 91.5% for blank CSNPs, with no significant difference from control ($p > 0.05$). The test concentration of 50 µg/mL used for subsequent work was therefore non-toxic.

4.12 Glucose uptake and cellular ROS

Palmitate exposure reduced insulin-stimulated 2-NBDG uptake to 54.3 ± 3.1% of normal control and increased intracellular ROS by a factor of 2.6, confirming successful induction of insulin resistance and oxidative stress (Table 7). Free extract partly restored uptake (71.2 ± 3.4%), similar to metformin (78.4 ± 3.6%). HE-CSNPs restored uptake to 92.8 ± 4.0%, significantly higher than the free extract at the same dose ($p < 0.01$) and approaching the normal control. Blank CSNPs had no meaningful effect (55.8 ± 3.0%). Intracellular ROS fell in parallel and was lowest with HE-CSNPs.

Table 7. Effect of treatments on insulin-stimulated glucose uptake and ROS in insulin-resistant L6 myotubes

Group	Glucose uptake (% of normal)	ROS (% of IR control)
Normal control	100.0 ± 4.2	38.0 ± 3.1
Insulin-resistant (IR) control	54.3 ± 3.1	100.0 ± 5.0
IR + metformin (1 mM)	78.4 ± 3.6	71.0 ± 4.2
IR + blank CSNPs	55.8 ± 3.0	96.4 ± 4.8

IR + free extract (50 µg/mL)	71.2 ± 3.4	68.0 ± 3.9
IR + HE-CSNPs (50 µg/mL)	92.8 ± 4.0	47.0 ± 3.5

5. Discussion

5.1 Formulation behaviour

The factorial results show that size and uniformity in chitosan–TPP systems are controlled by the balance between available amino groups and crosslinker. A chitosan:TPP ratio of 4:1 gave the most compact particles at each polymer concentration, whereas excess TPP (3:1) gave larger and more polydisperse populations, as is generally reported for ionic gelation [13,14]. The finding that entrapment was highest at an intermediate chitosan concentration indicates a trade-off between network density, which retains the extract, and viscosity, which hinders uniform particle formation. This interaction would have been missed in a one-factor-at-a-time approach and supports the use of a factorial design. A particle size of about 190 nm and a strongly positive zeta potential are within the range considered favourable for oral and mucosal delivery [11,12].

5.2 Structure and physical state

The FTIR shift of the chitosan N–H band and the appearance of phosphate bands confirm that TPP crosslinking, not mere physical mixing, formed the particles. The absence of new bands attributable to covalent bonds, together with preserved aromatic and phenolic absorptions of the extract, shows that the phytoconstituents were entrapped without chemical alteration. This matters for phytomedicine because chemical modification of marker compounds would change both activity and regulatory classification. Amorphization seen by XRD and DSC suggests molecular dispersion, a state usually associated with improved dissolution and higher biological activity of poorly soluble constituents, and the higher decomposition temperature of the encapsulated extract indicates a protective effect.

5.3 Release behaviour

The biphasic release profile, a small burst followed by prolonged release, is typical of matrix systems. The burst reflects surface-associated extract, which may be useful in practice because it provides an initial loading dose, while the remaining extract is released slowly by diffusion and swelling. An exponent of n = 0.47 places transport between Fickian diffusion and case-II transport, so that both polymer relaxation and diffusion contribute [20,21]. Sustained release of this kind is expected to extend the residence time of active constituents and flatten peaks and troughs in exposure, which is relevant for chronic conditions such as T2DM that require continuous dosing.

5.4 Why nanoencapsulation improved antioxidant potency

At equal extract concentration the nanoparticles were about 1.6 times more potent than the free extract in radical-scavenging assays. Several factors may contribute. Encapsulation can protect polyphenols from oxidation during storage and incubation, so more active compounds remain at the time of measurement. Dispersion at the nanoscale gives a high surface area and gradual exposure of phenolics to the radical solution. Chitosan itself has weak scavenging activity through its amino and hydroxyl groups, but the blank particles were almost inactive in our hands, so a synergistic contribution from the polymer is small. These findings agree with the general observation that nanoformulation enhances the apparent activity of phytochemicals, although a part of this effect in cell-free assays is a kinetic artefact of release and should be interpreted with care [9,16].

5.5 Mechanistic interpretation of the insulin-sensitizing effect

The larger recovery of glucose uptake with HE-CSNPs than with the same dose of free extract (92.8% vs 71.2%) is the central biological finding of this work. Three non-exclusive mechanisms are plausible, and each is testable:

1. **Greater intracellular delivery.** The positive surface charge promotes binding to the negatively charged plasma membrane and endocytic uptake, so more extract reaches the cytosol than by passive diffusion of the free extract.
2. **Relief of oxidative stress.** The fall in ROS (to 47% of IR control) removes a major inhibitor of IRS-1 signalling and Akt activation, as predicted by the oxidative-stress model of insulin resistance [4,5]. Activation of Nrf2-dependent antioxidant genes could prolong this effect [24].
3. **Direct signalling effects.** Gymnemic acids, trigonelline, diosgenin and flavonoids have been linked to AMPK activation and PI3K/Akt-dependent GLUT4 translocation [6,22,23]. Sustained release may maintain signalling

intermediates above threshold for longer than a single bolus of free extract.

The present assays measure the functional outcome, uptake and ROS, but not the pathway. Phosphorylation of AMPK, IRS-1 and Akt, and GLUT4 translocation, should be assessed by western blot or immunofluorescence to assign the mechanism. The lack of effect of blank nanoparticles shows that the carrier alone is not responsible for the response.

5.6 Comparison with metformin

At 50 µg/mL of extract, HE-CSNPs produced a greater recovery of glucose uptake than 1 mM metformin in this model. This should not be over-interpreted: the comparison is between a crude multi-component preparation and a single drug at a physiological in vitro concentration, and metformin acts in part through hepatic mechanisms not captured in muscle cells. It does indicate that nanoencapsulation can bring a herbal extract to a level of activity that is worth pursuing in animals.

5.7 Limitations

Several limitations should be acknowledged. Entrapment and release were quantified using total phenolics, which is a class-level marker; analysis of specific compounds such as gymnemic acids, trigonelline or diosgenin by HPLC would give a more reliable standardization. Dialysis release studies at a single pH do not capture the effect of gastric acid, where chitosan dissolves and swells, so release in simulated gastric and intestinal fluids should be examined. The biological work used a single cell line and a single concentration for the functional assays. Finally, in vitro uptake does not predict intestinal absorption, mucus penetration or first-pass behaviour, which require Caco-2 permeability studies and animal pharmacokinetics.

6. Conclusion and Future Perspectives

Chitosan nanoparticles loaded with a *Gymnema sylvestre*–fenugreek hydroalcoholic extract were prepared by ionic gelation and optimized by a 3 × 3 factorial design. The optimized formulation (chitosan 2 mg/mL, chitosan:TPP 4:1) had a size of 187.6 nm, a narrow size distribution, a positive zeta potential of +32.4 mV and an entrapment efficiency of 78.6%. Spectroscopic and thermal analysis confirmed ionic crosslinking and amorphous, molecular dispersion of the extract. Release was sustained for 48 h by an anomalous transport mechanism, and the dried powder was stable for 90 days at 4 °C.

Compared with the free extract, the nanoparticles had about 38% lower DPPH and ABTS IC50 values and restored insulin-stimulated glucose uptake in insulin-resistant myotubes to 92.8% of normal, with a parallel reduction in intracellular ROS and no cytotoxicity at the tested doses. Together, these results indicate that nanoencapsulation increases the antioxidant and insulin-sensitizing performance of the polyherbal extract.

Future work should proceed in four steps:

1. HPLC standardization of marker compounds (gymnemic acids, trigonelline, diosgenin) and measurement of their individual entrapment and release.
2. Release in simulated gastric and intestinal fluids, mucoadhesion tests and Caco-2 permeability studies.
3. Mechanistic studies of AMPK, IRS-1/PI3K/Akt and GLUT4 translocation, and of Nrf2 target genes.
4. Pharmacokinetic, acute and sub-acute toxicity, and efficacy studies in a high-fat-diet/low-dose streptozotocin rat model of T2DM, with oral glucose tolerance and HOMA-IR as primary endpoints.

If these studies confirm the in vitro findings, the formulation could be developed into an oral dosage form such as an enteric-coated capsule, with scale-up and regulatory evaluation as a standardized phytopharmaceutical.

Declarations

Data note. The experimental values in Section 4 are representative and must be replaced with the authors' own measurements before submission. References were compiled from the published literature and should be checked against the original sources (volume, pages, DOI) and the target journal's style.

Conflict of interest. The authors declare no conflict of interest.

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